PHYSIOLOGY OF METAL ION-INDUCED EFFECTS ON GERMINATION AND SEEDLING GROWTH IN CEREALS

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ABSTRACT

The effects of zinc, lead and copper ions, at concentrations up to 10 mM, on the germination and seedling growth of wheat, barley and rice were investigated. The percentage germination was unaffected by lead and zinc ions in the three species tested. The highest concentration of copper ions (10 mM) decreased the percentage germination in wheat, and totally inhibited germination in rice. Rice seedlings were grown in controlled environment cabinets using a nutrient culture technique. A copper ion concentration of 8 μM in the culture solution was found to inhibit the elongation of the longest root. Seedlings were grown in nutrient solutions at pH 5.5, 5.0 and 4.5, and three concentrations of copper ions. The root lengths were shortest at pH 4.5 , however, copper ion-induced inhibition in root elongation relative to the respective controls, was greatest at pH 5.5. Significantly more K^+ leaked from seedlings roots incubated in 4 μM copper ion solution than from seedling roots incubated in water. Similarly, the effect of copper ions on root lipid peroxidation as measured by TBA-rm (2-thiobarbituric acid-reactive material) accumulation, showed that more lipid peroxidation took place in copper ion treated roots than in control roots. The effect of various copper ion concentrations on the morphology and ultrastructure of the roots was studied using light and scanning electron microscopy. The results showed that copper ions caused a reduction in the length of the root hair zone and of the length of the root hairs, a reduction in the size of the epidermal cells of the root and a reductionin the distance from the root tip to the youngest lateral root.

The inclusion of 10 mM calcium, 8 mM magnesium, 50 μM citrate or 50μM oxalate ions in the nutrient solution ameliorated the toxicity of copper ions, however, seedling growth was never completely restored to that of the seedlings which were grown in the control nutrient solution. When roots were incubated with solutions of any of the ameliorants, the amount of potassium leaked from roots was 50 % less than when the external medium contained the same amount of copper without the ameliorant. The effect of calcium and magnesium on the accumulation of lipid peroxidation products in the roots appeared to be synergistic with that of the copper ions.

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DECLARATION

This thesis has been composed by myself and the work of which it is a record has been carried out by myself. All sources of information have been specifically acknowledged by means of reference.

Tariq Mahmood

DEDICATION

To the Agonies and Ecstacies of being a PhD Student.

CONTENTS

1. INTRODUCTION

The cereals belong to the Gramineae family and are the world's most important food crops. World food supply relies almost entirely on only 15 plant species and cereals comprise more than half of these. Approximately 700 million hectares of the earth's surface is devoted to cereal crops yielding more than 1800 million metric tons. Rice is the second most important in terms of the total production of cereals (Table 1.1.). More than 90 % of the world's rice is produced in Asia (FAO 1993).

Cereal crops	Area harvested (million hectares)	Production (million metric tons)
Wheat	219	564
Rice	148	518
Maize	127	477
Barley	73	161
Sorghum	44	61
Oats	20	32
Rye	12	29
Millet	37	26
Other	13	20
Total	693	1888

Table 1.1. World production of cereal crops, 1993 (FAO 1993).

Cereals are the dietary mainstay for the majority of the world's people. They are widely used as a measure of food production as over 50 % of dietary energy and protein in the human diet on a world basis is met from them. Demand for cereals is increasing enormously as the increasing population needs an adequate supply of food. Cereal products in Pakistan account for over 75 % of the human energy supply out of which more than one quarter is met by rice (FAO-Fifth World Survey 1987). Rice is the principal component of the human diet in most tropical countries. Half of the world's rice is produced on irrigated lowlands, whereas in Pakistan the entire rice crop is grown on canal-irrigated lands. During 1993, rice production in Pakistan was about 13 % less than it was in 1981 despite an eleven percent increase in the area under rice cultivation (FAO 1993). A number of environmental stresses such as drought, water-logging, salinity, metal ion pollution, diseases and insect pests are the main factors thought to be responsible for this yield drop. At present, although acute cases of land contamination by metal-containing materials attract a lot of attention and

pose a major risk to present agriculture, very little attention is given to the problems of metal pollution of agricultural land, especially in developing countries. It is already known that land erosion, excessive use of pesticides and intensive monoculture have caused considerable damage to productive lands and a decrease in the yield of rice and wheat in Pakistan, Nepal, Bangladesh and India (Mackenzie 1994). The effects of toxic metal ions on plant growth has become a topic of increasing interest. Although much information has been accumulated, most applies only to ecotypic species. Little information is available on effects of metal ions on crop plants.

1.1. Concept of "Heavy Metals"

Metal ions are directly or indirectly involved in plant metabolism and growth. Some are essential for normal plant growth e.g. potassium, sodium, magnesium, calcium, manganese, iron, copper, zinc, cobalt and nickel, while others have no apparent function e.g. aluminium, cadmium, lead, arsenic, chromium, mercury, and silver. A common characteristic of metals in general, regardless of whether they are essential or not, is that in excess they inhibit normal plant metabolism and thereby growth (Lanaras *et al.* 1993). The term "heavy metal", although not easily defined, is widely recognised and used as a group name for the metals and metalloids associated with pollution and toxicity. Pollution was defined by Holdgate (1979) as "the introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living resources or amenity, or interference with legitimate uses of the environment". Toxicity refers to the impairment of normal biochemical and physiological functions of living organisms. The existing, rather vague, classification of heavy metals is based on density (> 5 g cm^{-3}). This includes about sixty-five metallic elements, with the general ability to exert toxic effects on plant growth (Gadd 1992). It is a disparate group of metals and includes elements with diverse physical, chemical and biological properties, and is not a satisfactory method of classification in the present context. There are no specific chemical and physical properties that link these metal ions together. However, different scientists follow different conventions in defining these metals and so there is a current move to abandon the non-descriptive term "heavy metals". The best way to avoid confusion in the classification of these metals is to eschew the term "heavy metal" and simply state the specific metal ions concerned (Niedoer and Richardson 1980).

1.2. Background

It has been estimated that up to one fourth of the world's soils may inhibit the growth of crop species (Clark 1982). Soil is a vital ecological and agricultural resource and needs to be protected against further degradation. Soil contamination by toxic levels of metal ions can occur both through natural processes and as a result of man's activities. Man-induced addition of toxic metals such as copper, nickel and lead to the soil, through different sources, has been quite extensive and is likely to continue. This situation is of growing concern because these metals, unlike most organic pollutants which ultimately decompose, once added to the soil may persist for hundreds or thousands of years (Giller and McGrath 1989). The reason for this is that they are fairly immobile and cannot be broken down or leached from near-neutral topsoil or recovered from arable land by using current technology. However, their speciation might change with time as the organic molecules binding them decompose as soil conditions change. A small amount can leach away under very acidic or alkaline conditions but these conditions also increase the toxicity of the metal ions.

Soils act as a sink for metal ions due to several adsorption processes which bind them with varying strengths to the surfaces of the colloidal constituents of soils. The extent to which metal ions could be locked up in the soil depends upon a number of factors including the properties of the metals concerned, the pH, the amount of organic matter present, the CEC (cation exchange capacity) and the redox potential. Such adsorption of metal ions therefore delays or prevents the leaching down of metal ions through the soil profile or reduces their bioavailability to plants. For example, soils rich in clays, organic matter and base cations can cope with high inputs of toxic metals. On the other hand, sandy soils, which are acidic and have less organic matter and smaller CEC value, reach saturation point much more rapidly. Each soil type has a certain maximum metal ion adsorption capacity and cannot be used as an unlimited sink for toxic metals. Soils which have received a certain amount of metal ions but are not saturated to the extent that they show toxicity to normal plant growth are often considered suitable for crop production. This is a major oversight, because the adsorption capacity of soil is not constant and any change in the physico-chemical properties of the soil brought about by human activity or natural changes in the environment could tip the balance in an unpredicted way. This may convert slightly contaminated soils to highly toxic soils (Stigliani and Salomons 1993).

It has been estimated that the toxicity of all the metals, being released annually into the environment, far exceeds the combined total toxicity of all radioactive and organic wastes. The estimated annual global additions of copper, nickel and lead to the soil are 954,000, 325,000 and 796,000 tonnes, respectively (Nriagu and Pacyna 1988). Since the middle of the last century, the production and discharge of toxic metals to the environment has been increasing logarithmically (Nriagu 1979). Therefore, the consequences of toxic metal pollution of terrestrial ecosystems can be widespread and long-lasting. Nriagu (1988) termed this ever-increasing amount of metals entering into the biosphere as a "silent epidemic of environmental metal poisoning". The anthropogenic inputs have now overwhelmed the natural biogeochemical cycles of toxic metals in many ecosystems.

Anomalously higher concentrations of toxic metals found in agricultural soils can originate from a wide spectrum of natural and anthropogenic sources. This can occur from the agricultural use of fertilizers and pesticides, the addition of large amounts of livestock manures and sewage sludges to agricultural land, and other types of wastes. Intensive agricultural systems are highly dependent upon a substantial input of a variety of agrochemicals, many of which contain toxic metals. For example, commercial phosphatic fertilizers contain varying amounts of metal ions, depending on the rock source. Fertilizers applied over the last 100 years in North Carolina have contributed an appreciable amount of cadmium, chromium, nickel and zinc to the soil (Andriano 1986). At Rothamsted in the UK, the average annual increase of cadmium in wheat field soils has been estimated as 4.5 g ha^{-1}, since the late 1800s (Jones *et al.* 1988).

Land disposal of municipal effluents, sewage sludge, and animal wastes is widely practiced and is a major source of contamination of agricultural lands. In England and Wales, 52.9 % of total sewage sludge is disposed of on agricultural land and 500,000 tonnes is composted for use in UK agriculture annually. This is creating, potentially, an even worse pollution hazard than that attributed to fertilisers (Attwell 1993). It is estimated that each year from 4,900 to 21,000 tonnes of copper, 5,000 to 22,000 tonnes of nickel and 2,800 to 9,700 tonnes of lead is added to the soil, world-wide, as a result of disposal of sewage sludge (Nriagu and Pacyna 1988). Khan *et al.* (1993) reported increases over the past 20 years in the amount of copper, cadmium, and lead from 2-4.6, 7.23-19.3, and 0.7-1.4 times, respectively, due to past application of sewage effluent on the agricultural lands surrounding the city of Faisalabad, Pakistan.

The phenomenon of metal pollution is likely to become more devastating in developing countries than in developed countries (Nriagu 1990). In developing countries, crop growing areas in the vicinity of cities often receive rubbish as organic manure heavily loaded with toxic metals. In addition to that, sewage of the cities, one of the potential sources of metal pollution, is either used for growing crops and vegetables around the cities or drained out in irrigation canals. When sewage sludge is applied to soil, the beneficial effect, for example, due to the extra organic matter, N or P supplied, may be short-lived. However, the toxic metal ions introduced with them persist and accumulate with repeated applications. In Pakistan, there is no sewage treatment plant and sewage effluent is disposed off on agricultural lands except in coastal cities which drain their sewage effluent into the ocean. For crop production, the use of canal-irrigation receiving industrial wastes, poses a big threat to arable crop production in many parts of the world. In Pakistan about three fourths of the cultivated land is canal irrigated. Most of this receives sewage and industrial waste. Similarly, 16,000 ha of rice-growing area in Spain is bordering Albufera lake and is irrigated for rice production by its water, heavily contaminated by cadmium and nickel (Moragues *et al.* 1988). A few biocides contain copper salts and lead arsenate and mercury derivatives and have been used on a large scale. This practice still continues in many developing countries. There is no information available about the extent of contamination in developing countries. In addition to this, lax or ineffectual environmental control has encouraged some multinational companies to relocate their industries to the developing countries where they can also employ manufacturing processes that liberate hazardous metallic wastes and use materials that are banned in developed countries (Nriagu 1990).

In developing countries, the combination of population growth, economic pressure and the lack of government regulations can lead to an increase in the addition of toxic metal ions into the environment. In these countries, the majority of farmers have small enterprises, with low availability of inputs and resources. High socio-economic pressures compel the farmers to put all their efforts and inputs to highly productive lands, and crops are only occasionally grown on less favourable soils. Once the productive land also gets degraded or polluted due to salinity, water-logging or toxic metals, it is immediately taken out of general crop production and either used for some kind of afforestation, left uncultivated, or used for industrial or housing purposes where possible. Development of such land for housing or industry again results in the production of toxic wastes. Modifying soil conditions to suit crop

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agricultural lands, the use of heavy doses of fertilizers or lime is very expensive and consequently agriculture today flourishes only on the soils which are relatively easy to manage. Expanding food production to the less favourable soils to meet the demand generated by the ever-increasing human population is one of the main challenges to modern agriculture in developing countries. It demands an efficient means of metal toxicity assessment, amelioration of toxic soils, and the development of crops which can be grown successfully on polluted soils without yield loss or the incorporation of toxic metal ions into the food chain. It is therefore essential to attain a full understanding of the mechanisms leading to metal ion toxicity as well as those influencing tolerance in crop plants. This may enable us to regulate and routinely introduce tolerance into normally intolerant species. Such an ability has exciting implications in a world increasingly polluted with toxic metal ions.

1.3. Metal Ion Toxicity

Soils which have higher concentrations of metal ions than normal soils present an inhospitable environment for plant growth and development. Severe metal ion toxicity may cause complete impairment of normal biochemical and physiological functions leading to plant death. Visual symptoms caused by copper, lead (Pahlsson 1989) and nickel (Heale and Ormrod 1981) include stunted growth, chlorosis, necrosis, leaf epinasty, brownish-red discoloration, reduction in leaf size, and early leaf fall. Metal ion toxicity in plants has been investigated in a number of different ways. On the basis of these results it has been shown that short term survival tests using seedlings or vegetative propagules are very effective (Baker 1987). The first symptom of metal ion toxicity is the inhibition of root growth elongation. To study root growth in soil is technically very difficult because of the problem of isolating intact root systems. Furthermore, soil heterogeneity makes it difficult to isolate and study one factor at one time. Solution culture for metal ion studies has a number of advantages. It is simple to set up (plants are allowed to root in metal-amended and control solutions), rapid (frequently only a few days growth in treatment solutions is required), and easy to perform. There is a wide divergence of opinion about the application to the field situation of the results obtained from the solution culture method. However, Campbell and Lafever (1976) reported that solution culture test results and those from soil experiments have generally been found to be similar. Wong and Bradshaw (1982) used inhibition of root elongation of ryegrass grown in solution culture as an indicator of metal ion stress. They reported that this method was successful for the study of a number of metal ions. Karataglis (1980) criticised

the elongation method since it may be invalidated by the fact that the rate of elongation of the longest root tends to slow down with time. This is perhaps due to the growth of other lateral roots of the plant. He suggested that the measurement of all roots of the plant in test solutions and controls could be the best way to monitor plant response to metal ions. However, the findings of Rauser and Winterhalder (1984) did not agree with his suggestions. They found that the tolerance based on all of the roots was similar to that based to the longest root only. Despite all controversy about the methods for studying metal ion toxicity, most of the reported work has been done using nutrient/solution culture methods. Assays are based on the length of the longest root. In the subsequent section, observations made on some of the toxic effects of metal ions on different aspects of seed germination and seedling growth and development are reviewed.

1.3.1. Seed germination and seedling growth

Very often seeds can germinate in high concentrations of metal ions which could be toxic later in seedling growth. Wong and Bradshaw (1982) reported that the germination of *Lolium perenne* seeds was unaffected when germination was determined after 14 d of inclusion of various toxic concentrations of aluminium, cadmium, chromium, copper, iron, mercury, lead, manganese and zinc in the germination medium. Similarly, Singh *et al.* (1994) registered no effect of 1 mM copper on seed germination of *Sesamum indicum.* Nevertheless, the results of other studies show that there is a concentration of metal ions that will inhibit seed germination e.g. 100 mM copper completely inhibited germination in *Oryza sativa* (Gupta and Mukherji 1977). However, this is several times the concentration of metal ions found in toxic soils. It is possible that the insensitivity of the germination process to low concentrations of metal ions is due to the failure of ions to reach the internal tissues of the seed during imbibition. However, in the next stage of plant growth, which is generally called seedling growth and establishment, Gupta and Mukherji (1977) and Singh *et al.* (1994) found 100 μM and 1 mM copper ions caused inhibition in seedling growth of *Oryza sativa* and *Sesamum indicum*, respectively, when seedlings were grown on filter paper. Wong and Bradshaw (1982) attempted to find a relationship between the effect of metal ion concentration on inhibition of seed germination and seedling growth and development. They found no clear relationship between these two processes of growth as the concentration of metal ions inhibiting seedling growth showed no effect on germination. Normally, the concentrations of metal ions tested for germination studies are different from those

used for seedling growth, and the experiments are performed separately. However, it is not the process of germination but the growth and establishment of seedlings which is important because it is considered the most vulnerable stage in a plant's life (Forbes and Watson 1992) and populations of species that can tolerate toxic metal ions at this stage can survive the later growth stages (Sieghardt 1987).

Following successful germination, plant growth becomes more sensitive to metal ion toxicity. When 1 μM lead was present in the root medium of seedlings of *Hordeum vulgare* and *Zea mays*, Stiborova *et al.* (1986) found a significantly lower biomass and root/shoot ratio than in the control. Lanaras *et al.* (1993) found an inhibition in the growth of the wheat plants grown on ore bodies (48 mM copper in soil) with decreased height (25 %) weight (5 %) leaf area (7 %) and leaf dry weight (5%) compared to the control. Wheat and pea seedlings grown in nutrient solution containing elevated levels of cadmium and copper showed a reduction in relative growth rate compared to the control (Landberg and Gregor 1994). In general, all the toxic metal ions are known to reduce plant growth when present in excess of the normal concentrations. However the effect varies with the particular metal ion, growth stage and plant species concerned.

1.3.2. Root growth, development and morphology

Root growth is the most commonly assessed indicator of metal ion toxicity in plants, as the first signs of toxicity appear in root systems. These can become stubby, thick and discoloured (Karataglis *et al.* 1991). In *Zea mays*, a copper ion concentration as low as 7.5 μM in culture solution was shown to cause a 50 % reduction in root biomass of 15 d old seedlings compared to controls (Ouzounidou *et al.* 1994). Not surprisingly therefore, when 50 mM copper was applied to lettuce seedlings grown on soaked filter paper, root growth was completely inhibited (Mukherji and Gupta 1972). Root growth involves mitotic cell divisions which generate new cells, these new cells then elongate and increase in volume. The primary effect of toxic levels of copper (Wainwright and Woolhouse 1975, 1977; Hogan and Rauser 1981; Woolhouse 1983) nickel (Heale and Ormrod 1981) and lead (Godbold and Kettner 1991) on various species grown in solution culture has been shown to be on root elongation rather than on root initiation. However a decrease in the number of cells undergoing mitosis and a disturbed mitotic activity are also reported (Pahlsson 1989). Wong and Bradshaw (1982) and Ouzounidou *et al.* (1994) reported a 50% inhibition in root elongation of ryegrass and *Zea mays*,

respectively, at concentrations of Cu as low as 0.3 μM and 6.5 μM in culture solution. The process of inhibition of root elongation is thought to be initiated at the tip of the primary roots and subsequently at the tips of other branch roots (Wagatsuma *et al.* 1987). Biochemical and physiological studies to find out the process of inhibition of root elongation due to metal ion toxicity, suggested that it could be due to a change in the concentration of plant growth regulators e.g. decrease in the indole acetic acid content of the root, to changes in the processes of cell wall biosynthesis and assembly, and in the maintenance of positive cell turgor (Woolhouse 1983).

Root hairs are thought to be very important for the acquisition of mineral nutrients which in turn are delivered by diffusion to the root surface. It was concluded that their length and density are greatly affected by metal ion toxicity since no root hair formation was observed on the roots of rice seedlings when grown in nutrient solution containing 100 μM copper ions (Lidon and Henriques 1992 a). Similarly, Brady *et al.* (1993) reported a decrease in the root hair zone of *Glycine max* due to aluminium ion toxicity. They found failure, or slow emergence and poor development of root hairs after the inclusion of 2 μM aluminium in the nutrient solution. Some other morphological changes caused by aluminium ion toxicity on roots include a decrease in the organization of the peripheral cells of the root cap and in the cells of root surface in wheat (De Lima and Copeland 1994), and the destruction of epidermal and cortical cells in the tip and elongating region of maize root (Wagatsuma *et al.* 1987). In general, changes in root anatomy are closely correlated with changes in root morphology which result in reduced nutrient uptake due to the disturbance in uptake systems and/or to a reduction in root surface which ultimately impedes normal plant growth.

1.3.3. Photosynthesis and leaf anatomy

Lidon and Henriques (1991) studied photosynthesis in rice (*Oryza sativa*) leaves grown over a period of 30 d in nutrient culture containing a range of copper ions concentrations from 0.03 μM to 98 μM. They found a decrease in the rate of photosynthesis in plants grown in the higher copper ion treatments (98 μM) compared to controls. They attributed this effect to decreased concentrations of chlorophyll expressed as mg g^{-1} fresh weight of leaf. A similar effect of copper ion toxicity on photosynthesis of wheat plants was observed by Lanaras *et al.* (1993). They reported a decrease in the rate of photosynthesis of wheat plants grown on ore bodies (copper

3050 μ g g^{-1} dry weight of soil) compared to the plants grown in the same environment on control soils (2.21 mM copper in soil). The plants grown on the ore were chlorotic and showed a marked decrease (84 %) in chlorophyll concentration in comparison to controls. The inclusion of 2.5 and 5 mM lead in the nutrient solution decreased the chlorophyll content per unit leaf area and per unit leaf fresh weight of 28 d old barley plants in comparison to controls (Kacabova and Natr 1986). A comparison of the chlorophyll content of the leaves of sensitive and tolerant populations of *Silene cucubalus* grown for 30 days in nutrient solution containing 40 μM copper was made by Lolkema and Vooijs (1986). The chlorophyll content of the sensitive populations was significantly less (0.2 mg g^{-1} FW) compared to the tolerant populations (0.7 mg g^{-1} FW).

A reduction in chlorophyll content causing evenly distributed yellowing (chlorosis) over the surface of the leaf, is the characteristic symptom of metal ion toxicity. One of the postulated mechanisms of leaf chlorosis induced by an excess of copper ions, is the result of copper-iron antagonism which impedes uptake and translocation of iron (Kabata-Pendias and Pendias 1984). Lolkema and Vooijs (1986) and Lanaras *et al.* (1993) found no difference in the amount of iron in the leaves of plants grown on elevated levels of copper ions compared to normal levels in the root media. Wallace *et al.* (1977) suggested that it is the physiological utilization of iron inside the chloroplast, not the total concentration, which is affected by toxic metal ions and hence symptoms which are very much similar to those of iron deficiency are shown by the plant.

Another possible mechanism of leaf chlorosis involves acceleration of peroxidative degradation of lipids of the chloroplast membrane as a result of copper ion toxicity (Sandmann and Böger 1980). Lanaras *et al.* (1993) suggested that leaf chlorosis occurs as a result of metal ion toxicity in intact higher plants because two enzymes of the chlorophyll biosynthesis pathway, δ-aminolaevulinic acid dehydratase and protochlorophyllide reductase, are inhibited by metal ions. However, the mechanism causing leaf chlorosis caused by toxic levels of metal ions is not fully understood. In addition to metal ion-induced chlorosis of leaves, damage to the light reactions of photosynthesis has also been reported (Lanaras *et al.* 1993) especially inhibition of photosynthetic electron transport of photosystem II (Lidon and Henriques 1991).

Microscopy studies of transverse sections of leaves of wheat plants grown on copper-polluted soil showed a number of abnormalities which were attributed to the toxic effects of the high concentrations of copper ions in soil (Eleftheriou and Karataglis 1989). The mesophyll cells were more or less circular in shape whereas the equivalent cells of control plants were elongate to pleomorphic. In addition they reported relatively few, smaller and parietally distributed chloroplasts in mesophyll cells of plants grown on polluted soil compared to mesophyll cells of controls in which chloroplasts were abundant, large and crowded at the cell periphery. The chloroplasts of polluted plants contained a poorly-developed internal membrane system with only a few rudimentary grana whereas chloroplasts from control plants had an abundant internal membrane system organized in large grana.

1.3.4. Damage to plasmamembrane

The plasmamembrane regulates the passage of materials into and out of the cell, a function that makes it possible for the cell to maintain its structural and functional integrity. The capacity of a membrane to accomplish this function depends upon its composition and the properties of ions that interact. When there is a toxic amount of metal ions in the rhizosphere, the root cell plasmamembranes are an obvious initial site of action. It has been suggested that the primary toxic action of metal ions results in an alteration in plasmamembrane permeability. When the excised root segments of *Agrostis capillaris* (Wainwright and Woolhouse 1977) and *Mimulus guttatus* (Strange and Macnair 1991) or intact root systems of *Silene cucubalus* (De Vos *et al.* 1989) and wheat seedlings (Pandolfini *et al.* 1992) were incubated with elevated levels of metal ions, an increase in potassium ions in the external solution was observed compared to controls. These results suggested that metal ion toxicity causes an alteration of plasmamembrane permeability which results in ion leakage from roots and an influx of copper ions into roots from the external solution. This may lead to consequent loss of turgor and cell wall extensibility and/or to a disturbance in root metabolism which could bring about the arrest of root growth and subsequently tissue death.

The plasmamembrane is made up of a bilayer of lipid, in which embedded are large protein molecules. Approximately 40 to 50 % of the plasmamembrane is composed of lipids, mainly phospholipids, glycolipids and sulpholipids. The composition of the lipid bilayer differs between species and even between varieties of the same species and is altered in plants subjected to stress, for example treatment

with xenobiotics (Cooke *et al.* 1990) or adaptation to low temperature (Steponkus *et al.* 1990). Any environmental factor leading to a decrease in phospholipids (which are predominant) or to the oxidation of unsaturated fatty acids presumably causes changes in membrane integrity (Marschner 1986). A marked increase in lipid peroxidation products in the roots of *Silene cucubalus* was observed when they were treated with different reagents compared to controls (De Vos *et al.* 1989). The increase in lipid peroxidation products was highest with copper ions compared to other inorganic toxicants. In a further study De Vos *et al.* (1991) found that at higher concentrations of copper ions in the root medium of *Silene cucubalus,* the degree of root growth inhibition paralleled the rate of potassium ion leakage and the accumulation of lipid peroxidation products compared to controls. Pandolfini *et al.* (1992) concluded that lipid peroxidation could be one of the primary effects of nickel ions on plasmamembrane integrity as an increased concentration of malondialdehyde in roots (an indication of lipid peroxidation) was detected when potassium ion leakage was still at the control level. It is not only the peroxidation of lipids but the composition of fatty acids as well which is important for membrane integrity. De Vos *et al.* (1993) observed a significant decrease in fatty acid content, lower degree of fatty acid unsaturation and a greater accumulation of lipid peroxidation products in roots grown in copper solutions than in roots of plants grown in control solutions. They attributed these results to the peroxidative breakdown of unsaturated fatty acids as a result of copper ion toxicity. They suggested that lipid peroxidation is initiated by copper ions through catalysis of free radical production rather than through activation of lipoxygenase, an enzyme involved in lipid peroxidation. It is known that metal ion-induced lipid peroxidation causes impairment of membrane functioning, inactivation of membrane bound enzymes and non-specific permeability to ions (Gutteridge and Halliwell 1990). This ultimately results in decreased plant growth.

1.3.5. Effect on enzymes

Enzymes are among the first molecules with which toxic metal ions may interfere (Weigel and Jäger 1980). The result may be decreased activity of some or enhanced activity of others (Kar and Feierabend 1984). Two experimental approaches are generally used to study the interaction of metal ions with enzymes. For the *in vitro* method, plant proteins (enzymes) are extracted from plants grown on non-contaminated media. Different metal ion concentrations are then supplied to this extract. After a short incubation period enzyme activity is measured and compared to the control which contain no metal additions. In the *in vivo* method, plants are

grown on media substituted with or without (control) metal ions. Proteins (enzymes) are then extracted from the plants after a specific growth period and their activity is measured and compared to controls.

Enzyme inhibition

The plasmamembranes contain ATPases which are potentially metal-sensitive enzyme systems. These enzymes generate the driving force for transporting solutes into cells, and are considered important for growth and development and for adaptation to environmental conditions(Serrano 1989). Ros *et al.* (1992) observed an inhibitory effect on the membrane-bound ATPase activity of roots and shoots of *Oryza sativa* plants *in vitro* when 0.1 and 0.5 mM nickel and cadmium were included in the growth medium, compared to the respective controls. This reduced activity was correlated with decreased uptake of nutrients. Once nutrients are taken up into the roots, then the enzymes which are involved in internal utilization and transport become important. Nitrate, one of the major nutrients, is reduced inside the roots by the activity of nitrate reductase, a key enzyme for nitrate utilization especially in cereals. Relative to controls, a decreased activity of nitrate reductase in the roots of wheat seedlings grown in quartz sand amended with 25, 50, 100 and 150 mM nickel, lead, cadmium and copper was found by Bhandal and Kaur (1992). When toxic metal ions reach the leaf tissues they may then inhibit the activity of certain enzymes which are directly or indirectly involved in the process of photosynthesis, probably the most sensitive process of plant metabolism (Van Assche and Clijsters 1990). A drastic decrease in the activity of ribulose-1, 5-bisphosphate (RuBP) carboxylase and phosphoenolpyruvate (PEP) carboxylase in the leaves of maize and barley was found to be due to the effect of cadmium, copper and lead ions *in vivo* (Stiborova *et al.* 1987). RuBP carboxylase extracted from leaves of barley was found to have reduced activity as a result of cadmium *in vitro* in comparison to controls (Stiborova 1988). The biosynthesis of chlorophyll is also affected by metal ion toxicity, since the *in vitro* activity of one enzyme involved in chlorophyll synthesis, δ-aminolaevulinic acid (ALA) dehydratase, was found to be inhibited due to the toxic concentrations of lead and mercury ions (Prassad and Prassad 1987). Almost all the enzymes of plant metabolism are sensitive to metal ion-toxicity. However, the degree of susceptibilty differs amongst species, growth stage, environmental conditions and the metal ions concerned. Decrease in the activity of enzymes results in lowered or disturbed metabolism and this will affect the normal growth of the plant.

Enzyme induction

As the concentration of toxic metal ions increases in the cell, a stage is reached when the cell is irreversibly damaged. This stage may be reflected by an increase in the activity of certain enzymes in a phenomenon called enzyme induction (Van Assche and Clijsters 1990). Enzyme activity in plants is also induced by a variety of stress factors e.g. air pollution (Decleire *et al.* 1984), chilling (Levitt 1972) and pathogenic infection (Van Loon 1986). Enzyme induction has been observed in roots and leaves of various species after application of toxic doses of zinc, cadmium, copper, nickel and lead. Mukherji and Gupta (1972) studied enzyme induction in seedlings of *Lactuca sativa* grown in nutrient medium supplemented with varying levels of copper ions. They found a significant induction of catalase, peroxidase (POD) and IAA-oxidase in roots. A similar increase in root IAA-oxidase activity along with an increased concentration of copper ions in roots and a decreased root growth in *Hordeum vulgare* in comparison to controls, was reported by Coombes *et al.* (1976). POD is involved in the degradation of indole acetic acid (IAA). As a result, increased activity of IAA-oxidase due to copper ion toxicity in *Lactuca sativa* and *Hordeum vulgare* seedlings was thought to be significant for the growth inhibition of seedlings. Different isoperoxidases could also be induced by metal ion toxicity in higher plants. When *Oryza sativa* was treated with toxic amounts of zinc, copper and mercury the electrophoretic pattern of leaf extracts was changed as compared to the extract of control plants, showing some new isoperoxidases (Nag *et al.* 1981). In general, enzyme induction in plants can be considered as an indirect effect of toxic metal action. Some of the enzymes shown to be induced or to be synthesised in increased amounts, as a result of toxic metal ion treatment of the plant are enzymes known to be involved in stress metabolism.

1.4. Physiological Basis of Metal Ion Tolerance

Although metal-contaminated soils drastically decrease growth of plants and soil-living organisms, they are rarely devoid of flora and fauna. Many plants have evolved ecotypes or varieties that are able to grow and reproduce more-or-less normally on soils rich in toxic metal ions. These ecotypes are described as metal- tolerant. Ecotypes of the same species which are unable to survive on metal-rich soils are described as metal-sensitive or non-tolerant. Generally we can divide plant species into two groups; those which have been growing on normal soils for hundreds of years and the others, which are found both on and away from soils having toxic levels of metal ions. In the latter case, the species have evolved

ecotypes which are more tolerant to toxic levels of metal ions than the populations of the same species from normal soils. Certain ecotypes of species occurring on natural soils may possess some degree of metal ion tolerance. Understanding the mechanism of metal ion tolerance may help in developing cultivars of domesticated plant species which can cope with a widespread problem of metal ion pollution in soils (Macnair 1993).

A considerable literature has accumulated concerning the mechanisms of metal ion tolerance in plants, but regrettably, there is little information available about the metal-tolerance of cereals. Tolerance is defined as the presence of specific physiological mechanisms which collectively enable a plant to grow normally even in the presence of high concentrations of toxic metal ions (Baker 1987). There has been much speculation in the literature that different species may have different mechanisms of metal ion-tolerance. It is also thought that there may be further variation in the expression of tolerance to different metal ions within the same species or ecotype. Mechanisms of tolerance have yet been clearly elucidated. Postulated mechanisms of metal ion tolerance may be grouped into three categories (Fig. 1.1): (i) limited uptake of toxic ions or exclusion, (ii) compartmentalization of the metal ions within cells or within the plant and (iii) biochemical detoxification of the metal ions inside the plant (Berry 1986).

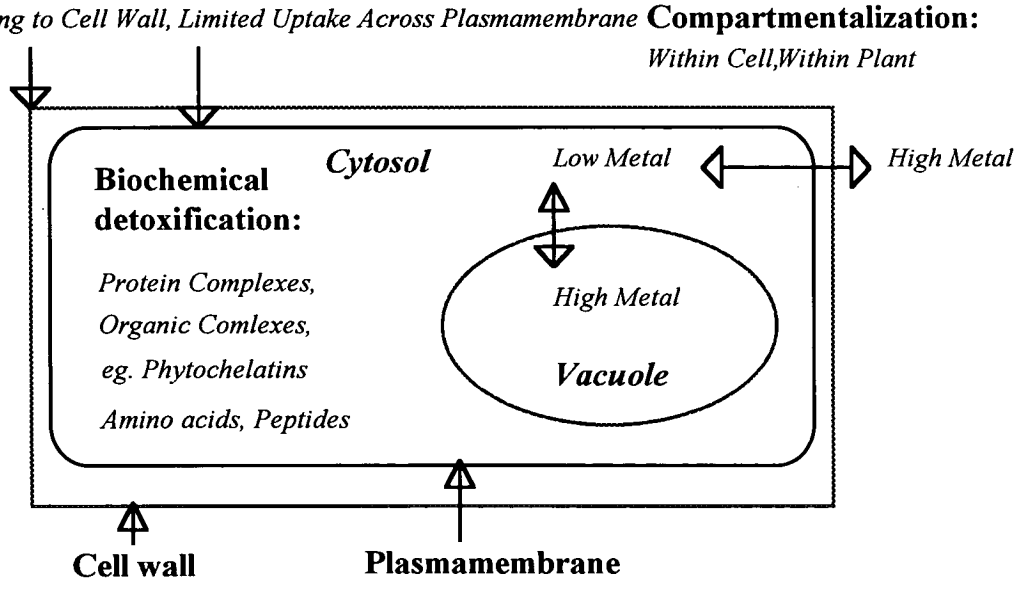


Figure 1.1. Possible mechanisms of metal tolerance in plants. Model adapted from Tomsett and Thurman (1988).

1.4.1. Exclusion

Plant cells are bounded by a cell wall. The uptake of ions across the plasmamembrane of root cells from an external solution first necessitates the ions traversing the cell walls in the root. In the exclusion mechanism, metal ions are thought to be prevented by the plant from entering into the root and reaching sensitive metabolic sites. Exclusion takes place either due to low cation exchange capacity at the cell walls in the root or due to precipitation of metal ions by hydroxyl ions at the surface of root plasmamembrane. In both cases, the uptake and absorption of metal ions into the cell is limited.

Immobilisation at the cell wall

The walls of the root cells are directly exposed to the metal ions in the soil solution. Association of metals with the cell wall has been frequently established, either through cation exchange techniques (Ernst 1969; 1972; Turner 1970; Farago and Pitt 1977), or by electron microscopical techniques (Ernst and Weinert 1972; Mullins *et al.* 1985). In the cell walls, metal ions are mostly bound to polygalacturonic acid (Ernst *et al.* 1992). Turner (1970) suggested that metal tolerance could be achieved by preferential accumulation of metal ions in the cell wall, resulting in reduced uptake of metal ions into the symplast. He found more zinc absorbed by the cell walls of the roots of zinc-tolerant clones of *Agrostis tenuis* than by the cell walls of the roots of zinc-sensitive clones, when both were treated with toxic levels of zinc ions. The interaction of metal ions with fixed negative charges such as carboxyl groups of pectate in the free space of the cell wall, results in selective binding of the cations. In turn, this may affect the ionic composition in the apoplast and subsequent ion translocation by the plant (Haynes 1980). A very strong copper retention capacity of the cell wall could be deduced from the findings of Jarvis and Robson (1982) who reported that at low copper concentrations in the solution, wheat, ryegrass and red clover can retain copper bound to root cell walls even when the shoots become copper-deficient. The extent of metal ion-binding by cell walls is considered to be a function of the metal ion exchange capacity of the cell wall and is related to the degree of tolerance to a specific metal ion. Localisation of lead and manganese in cell walls has also been demonstrated by microscopical and fractionation techniques (Memon *et al.*, 1980). Metal binding capacity of the root cell wall of *Athyrium yokoscense* showed that 70-90 % of the total root copper, zinc and cadmium was located in the cell wall (Nishizono *et al.* 1987). Similarly, Iwasaki

et al. (1990) also found that 67% and 60 % of the total root copper was bound in the root cell wall of Italian ryegrass and red clover, respectively.

However, the potential of the cell wall as a significant detoxification compartment in higher plants is questioned frequently (Verkleij and Schat 1990) because of the observed specificity of metal ion uptake and the differences in metal ion adsorption in tolerant and sensitive plants. Lolkeema and Vooijs (1986) found that when plants were grown in a solution containing 40 μM copper ions, copper concentrations in purified cell walls of the roots of sensitive genotypes of *Silene cucubalus* were four times higher than in the tolerant genotypes of the same species. However, when the metal ion concentration in the cell wall was expressed as a percentage of the total metal ion content, they found no difference between the two levels. They therefore concluded that metal ion storage in cell wall material did not play an important role in the tolerance mechanisms. The results of Cathala and Salsac (1975), who studied copper uptake in *Zea mays* and *Helianthus annuus* suggested that cell wall binding enhanced rather than inhibited copper uptake into the root. It appears, therefore, that immobilization of metal ions at the cell wall is not a general tolerance mechanism, but just one of the several mechanisms involved in metal ion tolerance. If cell walls do make a significant contribution to metal ion tolerance it is not known how the mechanism of metal ion-binding to the carboxyl groups of pectates in metal-sensitive plants is different from that in metal-tolerant plants.

Differential uptake at root plasmamembrane

It is conceivable that the plasmamembrane might play a role in metal ion exclusion by suppression of influx, increased efflux of solutes, preservation of membrane integrity or development of highly specific uptake systems which limit the transport of toxic ions. Growing roots can cause local pH and bulk density changes which affect metal convection and diffusion rates. Roots can alter the pH of the soil in the rhizocylender as much as several pH units. Changes in soil pH near the root can strongly affect metal movement to the root. Plants which maintained a relatively higher pH in the rhizosphere showed greater tolerance to aluminium than those which had a lower pH in their rhizosphere (Taylor 1987).

Suppression of the synthesis of an uptake system, or a conformational change in an uptake protein in the plasmamembrane has been discussed by Macnair (1990) as a mechanism of metal ion exclusion . He related such changes to reduced affinity of uptake sites or to a very high ion transport specificity. Similarly De Vos

et al. (1992) concluded that the restriction of copper uptake in *Silene cucubalus* at the plasmamembrane plays an important role in metal ion tolerance. All these theories of metal ion exclusion, however, have their shortcomings and do not fully explain the concept of sensitivity and tolerance and specificity of tolerance. It is unlikely that total exclusion of toxic ions will occur and therefore these mechanisms must be considered as limiting rather than preventing uptake (Meharg 1993).

1.4.2. Compartmentalisation

Compartmentalisation could provide a means of lowering the concentration of toxic metals at locations where the toxic effects are produced. Metal tolerance may be achieved by storing accumulated metal ions in organs or in subcellular compartments where no metabolic activity sensitive to metal ions takes place.

Within plant organs

Generally, plants accumulate higher concentrations of toxic metal ions in their roots than in their shoots. It was reported that about 96 % of whole plant copper was present in the roots of ryegrass growing in a medium with high available copper (Jarvis 1978). Baker *et al.* (1994) found a 10-100 times greater accumulation of lead, chromium, copper and iron in the roots of *Thlaspi caerulescens* than in the shoots. However, increased retention in the roots in tolerant plants is certainly not a rule (Baker 1981). It could be, however, of adaptive significance if shoots are more sensitive than roots, which is uncertain. Similarly, various plants are able to translocate metal ions into their old leaves before the leaves are shed. The oldest leaves of the metal-exposed plants generally exhibit the highest concentration of metals. In contrast to calcium, which accumulates in leaves gradually with ageing, zinc accumulates especially during the last week of shedding (Ernst *et al.* 1992), suggesting that plants make use of leaf fall as means of reducing their metal burden. Metal ion concentrations in reproductive parts are lower than in vegetative parts. In metal-tolerant plants, seeds have a lower metal concentration that any other plant organ (Ernst 1974, 1982). The concentration in the testa is often two to four times higher than in the embryo, which suggests that the placenta represents a barrier to transport of metal ions. Hibernating tissues and seeds often exhibit comparatively low concentrations of toxic metal ions (Ernst and Bas-Cramer 1980)

Within cells

At the sub-cellular level, the central vacuole would seems to be a reservoir for accumulated metal ions as it often contains high concentrations of zinc and nickel (Ernst 1969, 1972; Brookes *et al*, 1981) and to a lesser degree, copper and lead (Heuillet *et al.*, 1986; Rauser and Ackerley 1987; Vögeli-Lange and Wagner 1990). Ernst (1975) and Mathys (1975) postulated the zinc-malate-shuttle hypothesis for zinc transport through the tonoplast. Malic acid would bind the zinc in the cytosol, thereby detoxifying it, and the zinc-malate complex would be transported through the tonoplast and dissociated in the vacuole, after which the malate would be retransported into the cytosol. Vacuolar zinc would remain bound to stronger chelators, such as citrate, oxalate or anthocyanidins, when present. It has often been postulated that tolerance may be an increased ability to transport metal ions into the vacuole. This does not necessarily mean that tolerance would rely on a higher transport capacity. Ernst (1974) observed that leaf mortality in zinc-tolerant plants always occurred at the same fixed internal zinc concentration, suggesting that mortality is a consequence of a saturation of the vacuolar storage capacity. This does not mean, of course, that tolerance would be due to an increase of vacuolar storage capacity (Ernst *et al.* 1992). Transmission electron microscopy and x-ray analysis showed copper localisation within distinct structures, thought to be vacuoles (Robinson and Jackson 1986). Similarly, Rauser and Ackerly (1987) using transmission electron microscopy showed that cadmium is accumulated mainly in the vacuoles. By using compartmental flux analysis, Brookes *et al.* (1981), found that zinc-resistant clones of *Deschampsia caespitosa* were able to pump zinc actively into the vacuoles of root cells, whereas zinc-sensitive clones seemed to have a much lower capacity to do so. It seems that some transferase system at the tonoplast is involved in the differential storage of toxic metals. In summary, it is most likely that vacuolar compartmentation plays a role in tolerance to zinc and nickel, however, the actual mechanisms involved in intracellular compartmentalization are completely unknown (Verkleij and Schat 1990).

1.4.3. Biochemical detoxification

The mechanisms of metal ion detoxification in plants may involve enzyme adaptation in the form of altered enzyme sensitivity or induction of enzymes which lower the toxic effects of metal ions, and sequestration by phytochelatins.

Altered enzyme sensitivity

Generally, enzymes of metal-tolerant plants are as sensitive to metal ion stress as those of metal-sensitive plants. In some cases however, tolerance to metals can be associated with a low level of susceptibility of enzymes to metal ion inhibition. There is also a possibility of change in enzyme structure which could decrease metal-imposed loss of activity. Cox and Hutchinson (1980) demonstrated that root acid phosphatase from copper-tolerant *Deschampsia caespitosa* clones was less susceptible to copper inhibition than that from non-tolerant clones. Similar findings have been reported by Wainwright (1975) and Wainwright and Woolhouse (1975). An acid phosphatase, covalently linked to the cell walls of *Agrostis tenuis*, was regarded as a component of the extracellular compartment, and was shown to have a greater copper inhibitor constant in preparations from a copper-tolerant clone when compared to the enzymes from zinc-tolerant or normal pasture clones. However, the enzymes internal to the plasmamembrane were not found to show alteration in their properties in metal-tolerant clones (Cox *et al.* 1976).

Phytochelation

The toxic metal ion-binding peptides in plants are called phytochelatins. These are, apparently, the simplest (composed of only three different amino acids) natural compounds that may be involved in the detoxification and homeostasis of toxic metals. They do so through metal thiolate formation (Grill *et al.* 1985). It was demonstrated that phytochelatin synthesis is associated with glutathione or its biosynthetic precursors (Grill *et al.* 1986). The enzyme involved in the synthesis of phytochelatins, phytochelatin synthase, is produced by plant cells constitutively. Synthesis of phytochelatins by plants is strictly regulated by the availability of metal ions including cadmium, lead, zinc, antimony, silver, nickel, mercury, copper, tin, gold, bismuth, tellurium and tungsten. Phytochelatins are found in more than 200 species of higher plants and are produced in sensitive as well as tolerant plants (Steffens 1990).

The roots of copper-tolerant *Silene cucubalus* contained higher concentrations of polypeptide complexes than the roots of non-tolerant plants when subjected to toxic levels of copper ions (Lolkema *et al.* 1984). Similarly, it was found that copper-tolerant *Agrostis gigantea* synthesises these phytochelatins more rapidly than do copper non-tolerant plants of the same species (Rauser 1984). Therefore, the rate of metal complex formation may be an important determinant of tolerance. Robinson and Thurman (1985) found a phytochelatin copper complex in

the roots of copper-tolerant and non-tolerant *Mimulus guttatus* grown in sub-lethal concentrations of copper. The roots of the copper-tolerant plants contained more of the complex than the roots of the copper-sensitive plants. However, at higher external levels of copper ions, only 6% of the copper in the roots of the copper-tolerant plants was bound to this complex. This finding suggested that the mechanism of tolerance does not simply involve the sequestration of excess copper. Phytochelatins, probably have a transient function in the tolerance mechanism. It is proposed that the mechanism of copper tolerance in *Mimulus guttatus* could involve the binding of copper ions to a phytochelatin complex in the cytoplasm, and subsequent accumulation of copper in the vacuole. Clearly, if the complex is degraded in the vacuole, some of the copper is likely to be accumulated in this compartment. The amount of copper sequestered in such a pathway will depend upon the rate of turnover of the complex. Vogeli-Lange and Wagner (1990) found cadmium-binding peptides in the vacuole of tobacco leaves, and suggested that they might have a role in transporting the metal across the tonoplast.

As phytochelatin production can be induced by a range of metal ions, the role of phytochelation in metal ion tolerance is frequently questioned. It is becoming increasingly probable that phytochelatins are not involved in differential tolerance in tolerant ecotypes; rather that they are thought to have a general non-specific role in metal ion tolerance (Verkleij and Schat 1990).

1.5. Amelioration of Toxicity

Germinating seeds and developing seedlings are susceptible to metal ion toxicity (Patterson and Olson 1983) because, firstly, they are very tender at this stage and secondly, the toxic metal ions normally stay in the top soil layers where germination and seedling development take place. Once the seedlings have passed these stages with minimum deleterious effects of metal ions, they will have a good chance of producing a crop. This could be achieved either by growing seedlings which are tolerant of the levels of toxic metal ions in the soil, or by applying some amendments which minimise the toxicity of the metal ions. Much work has been reported about identifying tolerant plants and species which can be grown in soils with toxic concentrations of metal ions (Blum 1988). Reduction in the bioavailability of toxic metal ions in the soil is the most common method of soil amelioration. Conventionally, lime is applied to the soil to raise the pH to 7.0 or higher. This changes the solubility of metal ions and renders them less mobile and poorly available

to plants. This method has been practiced regularly in vineyards where copper toxicity occurs as a result of the accumulation of copper ions from fungicides. This involves large and repeated application of lime, and may not be effective for all metal toxicities (Clark 1982). Furthermore, over a period of time, lime may induce secondary toxicity or associated deficiencies. However, in many developing countries, liming the land on a large scale is not economically feasible. Therefore, this practice is of limited value as a practical solution to alleviating metal ion toxicity on a large scale and/or a permanent basis. An amendment which is required in small quantities and which has a plant-based application would be a better alternative. This is of much importance for crops which are transplanted at the seedling stage. The application of calcium (Rengel 1992; Brady *et al.* 1993) and magnesium (Tan *et al.* 1992 a) and of citrate and oxalate (Shuman *et al.* 1991) in solution culture, has been reported for the amelioration of aluminium toxicity. However, the effect of these ameliorants on copper ion-induced toxicity has yet to be investigated.

1.6. Aims and Objectives

Metal ion pollution of agricultural lands is becoming a problem in arable crop production. This situation is worsening day by day, especially in the developing countries, such as Pakistan, where rules and regulations to check the pollution of lands are not very strict. There is a dearth of information about the toxic effects of metal ions on crop plants. The aims of this project were therefore that:-

(i) To evaluate the effects of metal ions on seed germination and seedling growth in cereals.

(ii) To find out the minimum concentration of copper ions toxic to rice seedlings, and the relationship of copper ion toxicity to the pH of the culture medium.

(iii) To investigate some aspects of the physiological basis of copper ion toxicity in the roots of rice seedlings.

(iv) To evaluate the effects of toxic concentrations of copper ions on root morphology in rice seedlings.

(v) To evaluate the ameliorative effects of calcium, magnesium, citrate and oxalate ions on copper ion-induced changes in the growth and root physiology of rice seedlings.

2. MATERIALS AND METHODS

General materials and methods are described in this chapter whereas specific methods relating to each experiment are described in the successive chapters.

The experiments were carried out in Fisons Controlled Environment Cabinets (FISON, Fi-totron 600H) in the Plant Growth Unit of the Department of Crop Science and Technology, The Scottish Agricultural College, Edinburgh.

2.1. Effect of Metal Ions on Seed Germination and Seedling Growth

A series of germination and seedling growth tests were performed on wheat, barley and rice using $CuSO_4$, $ZnSO_4$, $PbCl_2$, $MgSO_4$ and NaCl. All chemicals were of AnalaR® grade from BDH Chemicals Ltd. Poole, England. Water absorbent paper, made by Kimberley Clark, Larkfield, Kent, England was supplied by Dr B.Rennie, Official Seed Testing Station for Scotland, East Craigs, Edinburgh. Seeds of wheat (*Triticum aestivum* L. cv. Tonic) and barley (*Hordeum vulgare* L. cv. Triumph) were supplied by Mr Robert Redpath, Crop Science and Technology Department, The Scottish Agricultural College, Edinburgh. Rice (*Oryza sativa* L. cv. NIAB 6 and cv. Basmati 370) seeds were supplied by Prof. R.H. Qureshi, Soil Science Department, University of Agriculture, Faisalabad, Pakistan.

All solutions were freshly prepared for each experiment. They were made up in ultra pure water prepared by passing tap water through a reverse osmosis membrane, carbon filter and ion exchange cartridge. Following purification of the water the metal ions were below detection limits (for detection limits see Appendix I) of the ICAP 61E, Inductively Coupled Plasma Atomic Emission Spectrometer (Thermo Jarrel Ash Corporation, Franklin, MA, USA). One litre stock solution of 100 mM was prepared for each metal ion which was used to make 500 ml of each subsequent concentration. Ultra pure water was used as control.

2.1.1. Germination Test

Disposable polystyrene Petri dishes 90 mm diameter (Bibby Sterilin Ltd., Stone, Staffs, UK) were washed with ultra pure water, then left overnight in 1% HNO_3 and rinsed twice with ultra pure water before setting up the experiment. Seed

samples were taken at random from a bulk of 0.5 kg and twenty five seeds were placed in each dish equally separated from each other between three layers (one at the top and two at the bottom) of filter paper (Whatman, Grade 181). To each petri dish, 10 ml of the ultra-pure water or treatment solution was added. Petri dishes were sealed with Parafilm and put in the dark in controlled environment cabinets at 20 ± 2 °C for wheat and barley and at 30 ± 2 °C for rice. Each treatment was replicated four times, and Petri dishes were completely randomised in the cabinets. After 96 h the seeds with 5 mm radicle length were taken as germinated.

2.1.2. Seedling Growth Test

For the assessment of seedling growth a modification of the method of Perry (1977) was used. A line was drawn on water-absorbent paper (300 x 80 mm) parallel to the long axis, mid-way between the longer sides. A sample of twenty five seeds was taken at random and seeds were glued to the paper on the line, 10 mm apart, using silicon Sealant glue (Evode Ltd. Stafford, England). The crease side of the grain was kept next to the paper, and the grain was placed with the embryo end towards the bottom of the sheet, and the coleoptile pointing upwards. Two additional sheets of paper were placed one on top and one below the paper bearing the seeds. The basal 20 mm of the paper was folded upwards, and the paper was rolled loosely and placed upright in a 250 ml (12 cm high and 6 cm in dia.) specimen tube (Bibby Sterilin Ltd., Stone, Staffs, UK). The specimen tubes had previously been washed in the same way as the Petri dishes (see section 2.1.1.). To each specimen tube, 100 ml of the ultra-pure water or treatment solution was added. The specimen tubes were placed in the dark in a controlled environment cabinet for 7 d at relative humidity (RH) > 90% and 20 ± 2 °C for wheat and barley and 30 ± 2 °C for rice. Drying of the sheets during incubation was prevented by topping up the specimen tubes with ultra-pure water. The four replications per treatment were completely randomised in the cabinets. Plumule length and the length of the longest root, and the number of roots were determined for all seedlings after 7 d of treatment.

2.2. Method development

In order to investigate the effect of metal ions on the seedlings beyond 7 d growth, a system was needed in which seedlings could be easily and frequently observed without any damage, the concentrations of treatment solutions could be kept constant and the seedlings could be supported to grow normally. For seedling

growth evaluation (2.1.2), seeds were placed or glued on germination papers and put inside specimen tubes or trays containing distilled water or nutrient solution (Perry 1977; Kaur and Duffus 1989). Rolled paper towels, when left in treatment solutions for a period of days, start to disintegrate and so do not give proper support to the growing seedlings. In addition, when the rolls are opened the roots get damaged because they penetrate the paper during growth. Germination papers are made of cellulose and have high metal-adsorbing capacity. This may produce considerable variation in the concentrations of ions to which the roots are exposed. It was found that the germination papers used for germination and growth tests, when left in 6 and 12 μM copper ion solution for 12 h adsorbed about 50 % of the copper ions, whereas non-adsorbent polyethylene sheets showed no adsorbance of copper ions (Table 2.1).

Table: 2.1. Concentrations of copper ions left in the solutions after incubation with different materials for 12 h.

Initial (μM)	After incubation with paper towel (μM)	After incubation with black polyethylene (μM)
6	2.70	5.97
25	13.45	24.80

Rice grains were sown in acid-washed neutral sand moistened with distilled water and left in the dark at room temperature. At the third leaf stage, 24 seedlings were transplanted into perspex trays (38 x 26 x 6 cm) per replication of each treatment. Each tray contained 3 L of Yoshida nutrient solution (Yoshida *et al.* 1976), pH 5.5 ± 0.1. The seedlings were inserted in the holes made in polystyrene tiles and were fixed with a thin layer of foam. They were then floated on nutrient solution. The trays were put in a plant growth room at 16 h light at 30 °C and 8 h dark at 25 °C, with 70 % RH. Photosynthetically available radiation (PAR) was 120 μE m^{-2} s^{-1}. However, the seedlings did not survive in the nutrient solution. Damage to roots and transplanting shock was considered to be responsible.

In order to avoid any damage to roots during transplanting, the grains were germinated in rolled paper towels moistened with distilled water and kept at 25 °C in the dark. After 4 d, germinated seeds were transplanted into nutrient solution in trays as described above. When the seedlings were 10 d, $CuSO_4$ was added to give 20, 50, 100 and 200 μM copper ion concentrations in the nutrient solution. The

nutrient solution without additional $CuSO_4$ was used as the control. After 10 d of treatment (when seedlings were 24 d old) seedlings were dying in the 100 and 200 µM copper treatments. At the same time, the top surface of the polystyrene sheets was heavily infested with algal growth. In the plant growth room it was very difficult to change the solution frequently and maintain the trays free of algae. On the basis of this experience it was considered that further work should be carried out in controlled environment cabinets where seedlings could be grown in liquid culture, supported in specimen tubes. Thus, solutions could be changed more frequently. As the copper ion concentrations of 100 and 200 µM were shown to be very toxic, further experimental work involved lower copper ion concentrations which were toxic enough to produce inhibitory effects but did not kill the seedlings.

As a result of these observations, the following procedure was developed. This was used in the experiments described in chapters 4, 5 and 6.

2.2.1. Experimental procedure

Black non-adsorbent polyethylene sheets 40 x 12 cm were used for rice seedling growth. Grains were fixed to the sheets through 2.5 mm parallel slits made apart in the sheet (Fig. 2.1).

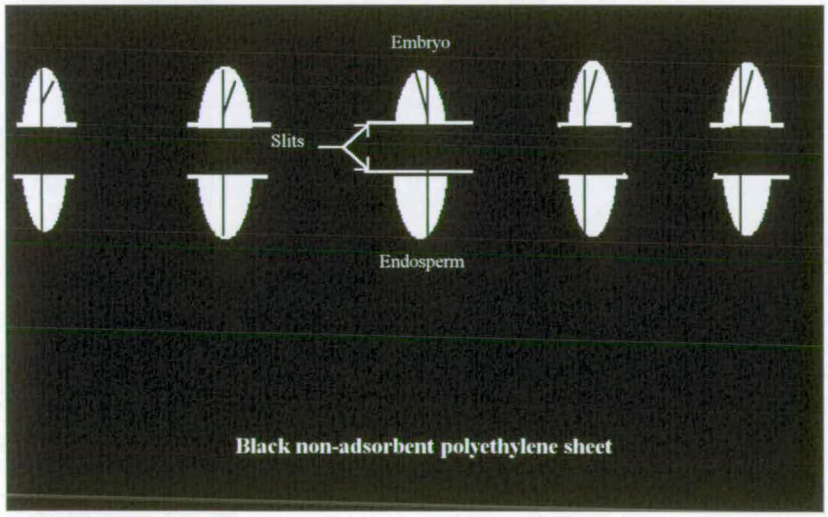


Fig. 2.1. Seeds fixed in the sheet without any glue, for seedling growth studies.

Fifteen grains were inserted in slits, equidistant from each other, 9 cm from the base of the sheet. The grains were stuck in such a way that the embryo was directed towards the top of the sheet, and endosperm towards the base of the sheet (Fig. 2.1). The seeds were inserted with the embryonic end towards the upper side of the sheet because after germination the roots which emerged changed their direction of growth by 180° and, by growing over the slit in the polyethylene sheet, they gave an anchoring support to the seedling. The sheets were rolled gently, with the grains inside the roll, and placed inside the specimen tubes containing approximately 240 ml of full strength Yoshida nutrient solution supplemented with different concentrations of copper ions. Full strength Yoshida nutrient solution was used as control. The solution level in the beaker was managed in such a way that the endosperm of the grain was submerged in the solution thus facilitating the process of imbibition while the embryonic part was in the air in order to allow oxygen uptake by the grains. The specimen tubes were placed for germination in a controlled environment cabinet at 27 °C, 60 % RH in darkness for 3 d. Thereafter, growth conditions in the cabinet were 16 h light at 30 °C and 8 h dark at 25 °C and RH 70 %. Irradiance was 100 μE PAR m^{-2} s^{-1}. The solutions in the specimen tubes were renewed every alternate day until the harvest.

2.3. Application of GEOCHEM-PC

The computer programme GEOCHEM-PC (Parker *et al.* 1994) was used to simulate ionic concentrations of different metal ions in control and treatment solutions. This model uses simple laws of chemical thermodynamics and is a practical means for predicting metal ion speciation in solutions.

2.4. Chemical analysis

At the end of the treatment period, seedlings were removed from the specimen tubes and their roots given three 5 min washes with distilled water and then blotted to remove surface moisture. The plants were separated into roots and shoots by cutting at the hypocotyl junction of the root to shoot. The samples were dried at 100 °C, to a constant dry weight, in a draught oven. The dried roots and shoots were digested separately in nitric acid:perchloric acid (4:1) digestion mixture (Thompson and Walsh 1989) and analysed for copper ions on an ICAP 61E, Inductively Coupled Plasma Atomic Emission Spectrometer. The results were expressed as μg g^{-1} (dw) of root or shoot.

2.5. Chlorophyll Measurement

Chlorophyll in the fully-expanded uppermost leaf of seedlings was determined by the method of Wintermans and De Mots (1965). 0.1 g of fresh leaf was homogenised in about 3 ml of 96 % ethanol at 70 °C. The material was centrifuged at 600 rpm in a centrifuge (CENTAUR 2E, Bench-Top Centrifuge, Fisons Scientific, England) for 15 min. The supernatant was transferred to a 5 ml volumetric flask and the volume of the liquid was made up to the mark with 96 % ethanol. Using a Beckman DU 65 spectrophotometer the absorbance at 654 nm was read against an ethanol blank. The concentrations of chlorophyll ($\mu g\ ml^{-1}$) was calculated using the following equations:

$$\text{Chlorophyll a} = 13.70\ A_{665} - 5.76\ A_{649}\ \mu g\ ml^{-1}$$
$$\text{Chlorophyll b} = 25.80\ A_{649} - 7.60\ A_{665}\ \mu g\ ml^{-1}$$
$$\text{Chlorophyll a + b} = 1000\ A_{654} / 39.80\ \ \mu g\ ml^{-1}$$

2.6. Trypan Blue Exclusion Test

The integrity of the plasmamembrane of the root cells was assayed using the trypan blue exclusion test adopted by De Vos *et al.* (1989). The seedlings were removed from the treatment solution with intact root systems. Keeping the longest root intact, all the branch roots were cut carefully while they were still bathed in the respective treatment solution in a petri dish. The longest root thus separated was placed in 0.5 % (w/v) trypan blue in water for 5 min. After gently washing with distilled water the roots were placed on a glass slide and covered by a cover slip. The roots were examined for dye staining using a dissection microscope (Kyowa Optical, Model SDZ, Finaly Microvision, England).

2.7. Potassium Leakage Measurement

Seedlings were removed from specimen tubes with the whole root system intact. The root systems were rinsed in distilled water and pre-incubated for 3 h in a test solution containing 0.5 mM $CaSO_4$, 0.1 mM KCl and 1.5 mM MES-Na^+ (2[N-morpholino]ethanesulfonic acid) at pH 5.5 (De Vos *et al.* 1989). During the incubation period, the specimen tubes were kept shaking on an orbital shaker at 70 rpm. The solution was changed once during the pre-incubation period. After 3 h

the seedlings were removed and the root systems were given three 5 min washes with distilled water to wash out potassium ions from the apparent free space.

After pre-incubation, 12 seedlings per treatment having apparently uniform root systems and with their whole root systems intact, were transferred to 50 ml of the treatment solutions prepared in distilled water. The whole root system was submerged in the treatment solutions contained in specimen tubes. The specimen tubes were put in a growth cabinet under light where temperature and humidity was same as for seedling growth. After a specified treatment period, seedlings were removed from the solution and roots were separated from shoots. The roots were blotted dry with paper tissue and the root fresh weight was measured. After the removal of the seedlings, the volume of the treatment solution in specimen tubes was made up to 50 ml by adding distilled water and the concentration of potassium ions present in the solution was determined using a flame photometer (Corning, 400).

2.8. Lipid Peroxidation Measurement

The amount of lipid peroxidation in intact roots was estimated by measuring the amount of TBA-rm (2-thiobarbituric acid-reactive material), formed in a given period of time. The TBA-rm material is present as aldehydes, mainly malonaldehyde, and as endoperoxide (Buege and Aust, 1978). After pre-incubation of seedlings and treatment applications as described in section 2.7., TBA-rm in root tissues was assayed according to the method of De Vos *et al.* (1991), using 0.25 g fresh weight of whole roots per 4 ml of 0.25 % TBA (2-thiobarbituric acid) in 10 % trichloroacetic acid (TCA). After heating at 95 °C for 30 min in a waterbath and subsequent cooling, the mixture was centrifuged at 6000 rpm for 15 min. The absorbance of the supernatant at 532 and 600 nm was measured using a Beckman DU 65 spectrophotometer. The difference between these two absorbances ($A_{532\ nm}$-$A_{600\ nm}$) was proportional to the amount of TBA-rm present. The blank was 0.25 % TBA in 10 % TCA.

2.9. Scanning Electron Microscopy

2.9.1. Cryopreserved specimens

After treatment, the branch roots of the seedlings were removed, while keeping the seedlings in the treatment solution in a Petri dish. The longest roots of

the seedlings were prepared for cryofixing by the method of Brady *et al.* (1993). Holding the shoot, the root system was plunged into liquid nitrogen (–196 °C) for 30 s. Each root system was frozen individually in this way. The roots were then cut from the shoot and transferred to super-cooled (–98 °C) absolute methanol for freeze substitution. After 3 d, approximately 70 % of methanol was replaced with pre-cooled fresh absolute methanol, without exposing the roots, and transferred to a –20 °C freezer for 3 weeks prior to critical point drying. The frozen-substituted roots were placed cold into a critical point dryer at –67 °C and flushed repeatedly with liquid carbon dioxide gradually replacing all methanol in the roots. The temperature of the sealed critical point drier was raised above the critical point for carbon dioxide (31 °C @ 1072 psi) and the gas was gently bled off to dry the specimens. The dried specimens were mounted on SEM specimen stubs using carbon adhesive discs and earthing was achieved by silver dag (Agar Scientific Ltd., 66 A Cambridge Road, Stansted) and sputter-coating with gold using argon gas (20 mA @ 0.1 Torr for 2 min). These samples were examined using a Cambridge Stereoscan S250 electron microscope and recorded on TMAX 100 35 mm roll film.

2.9.2. Cryosectioned specimens

Fresh root samples, were mounted in Tissue Tek (Agar Scientific Ltd., 66 A Cambridge Road, Stansted) and rapidly frozen (cryofixed) in liquid nitrogen (–196 °C). The frozen mounted samples were sectioned on a cryostat E (Reichert-Jung Freezing Microtome) at –30 °C. The sections were discarded and the remaining part was transferred to the pre-cooled stage of a Speedivac-Pearse freeze-dryer at –60 °C for 2 d. After that, the temperature of the freeze-dryer was gradually increased to allow sublimation of ice present in the specimens. These dried specimens were then mounted on SEM stubs, earthed with silver dag, sputter coated with gold and examined by SEM.

2.9.3. Replicas of specimens

Moulds of the longest roots of the seedlings were prepared using Extrude Wash dental impression material (Polyvinylsiloxane Impression Material, Type I, ISO Type IIIA, Kerr Manufacturing Company, Romulus, USA) following the method of Williams *et al.* (1987). In this procedure, equal quantities of base and catalyst were mixed thoroughly and spread in the form of a thin layer on glazed paper. Care was

taken that no air bubbles remained. After removing branch roots, while keeping the seedling in the treatment solution in a Petri dish, the longest root was washed with distilled water and blotted by gently placing between two layers of paper tissue for 30 s. The roots were placed on the impression material within 1 min of mixing it and the material was allowed to polymerise for 5 min at room temperature (20-22 °C). The roots were then removed and negative replicas obtained. Agar resin (Agar 100 resin kit, Agar Scientific Ltd., 66 A Cambridge Road, Stansted) was introduced into the moulds and polymerised overnight at 60 °C. The positive resin replicas were removed from the moulds and stuck to SEM stubs with carbon adhesive discs. The replicas were earthed with silver dag, sputter-coated with gold and examined under SEM.

2.10. Statistical Approach

For germination and seedling vigour experiments there were four replications for each treatment, whereas for seedling growth experiments each treatment was replicated three times. The design of the experiments was completely randomised design. The data were analysed for analysis of variance using 'Genstat', version 5.0 (Statistical Department, Rothamsted Experimental Station) and means were compared at 5 % level of probability by t-test.

3. EFFECT OF TOXIC METAL IONS ON SEED GERMINATION AND SEEDLING GROWTH IN CEREALS

3.1. Introduction

Dry seeds are capable of withstanding adverse environmental conditions. However, when they are hydrated and are in a favourable environment, the processes of metabolism, cell division and nutrient mobilisation start and they become sensitive to environmental stresses. Germination and seedling establishment are critical stages in the life cycle of a crop plant as they determine the density of the final crop stand. Toxic metal ions in soil, affecting seed germination and seedling growth have generally been found to reduce greatly the prospects of a good crop. It has been shown that the process of germination is fairly insensitive to the effect of toxic metal ions (Pahlsson 1989). At low metal ion concentrations, germination is merely retarded, and only at higher concentrations is the final percentage germination reduced. Percentage germination in pearl millet (Davis *et al.* 1993) and in wheat (Hasnain *et al.* 1993) was inhibited significantly by copper ions at a concentration of 5 mM, and lead ions at a concentration of more than 10 mM, respectively. Complete inhibition of germination in lettuce (Mukherji and Gupta 1972) and rice (Gupta and Mukherji 1977) by copper ions at 100 mM and by both copper and lead at 100 mM in maize (Leblová *et al.* 1986) was observed. However, these concentrations of metal ions which either inhibit germination or decrease percentage germination are not commonly found under natural conditions, except in places which are very heavily polluted by toxic metal ions, or in serpentine soils. This is perhaps the reason why the number of studies devoted to the effects of metal ions on seed germination is small compared to the number of studies on the effects of metal ions on seedling growth (Pahlsson 1989).

It is not the process of germination but the growth of the seedlings, which is the most sensitive stage in a plant's life (Forbes and Watson 1992). Growing roots and shoots are susceptible to metal ion concentrations which do not have any effect on seed germination. Wong and Bradshaw (1982) reported 95 to 100 % germination of *Lolium perenne* seeds when subjected to those concentrations of toxic metal ions which resulted in almost complete inhibition of root growth. They found no relation between the effects of metal ions on seed germination and the primary effects of the metal ions on root growth, because root growth was retarded at low concentrations of metal ions.

The response of different crop species to different metal ions is variable, and any plant species that can tolerate toxic metal ions at the seedling stage can survive the later stages of growth (Sieghardt 1987). There is a scarcity of information about the effects of toxic metal ions on the germination and seedling growth of cereals. The objectives of the present work were therefore (1) to determine the effects of different concentrations of copper, zinc and lead ions on germination and seedling growth of barley, rice and wheat; (2) to determine the effect of the anions of the metal salts used; (3) to find the minimum concentration of metal ions toxic to seedling growth in these cereals in order to determine the appropriate concentrations to use in further investigations on the physiological effects of metal ions.

3.2. Experimental Procedure

Germination and seedling growth of the seeds was assessed by the procedure described in Section 2.2.1 and 2.2.2 on wheat, barley and rice using solutions of $CuSO_4$, $ZnSO_4$, $PbCl_2$, $MgSO_4$ and NaCl in various concentrations. All solutions were freshly prepared for each experiment. They were made up in ultra-pure water, which was prepared by passing tap water through a reverse osmosis membrane, carbon filter and ion exchange cartridge. One litre stock solution of 100 mM was prepared in each case and was used to make 500 ml of each subsequent concentration. Ultra-pure water was used for the controls.

3.3. Results

Percentage germination

The effect of different concentrations of $CuSO_4$ and $MgSO_4$ on the percentage germination in barley, rice and wheat is shown in Fig. 3.1. The percentage germination in barley, rice and wheat at 0.1 mM and 1.0 mM $CuSO_4$ was statistically similar to that of the respective controls, though there was some inhibition in germination at 1.0 mM CuSO. In the 10 mM $CuSO_4$ treatment, the germination percentage in barley was the same as that of the control, whereas rice seeds failed to germinate and in wheat, germination was 47 % less ($p < 0.05$) than that of the control. To test whether or not the inhibition of germination was due to copper ions or to sulphate ions, the percentage germination was recorded in the same range of concentrations of $MgSO_4$. The percentage germination at all the concentrations of $MgSO_4$ was the same as that of the respective controls in the three species. In another experiment, the effect of $PbCl_2$, $ZnSO_4$ and NaCl was

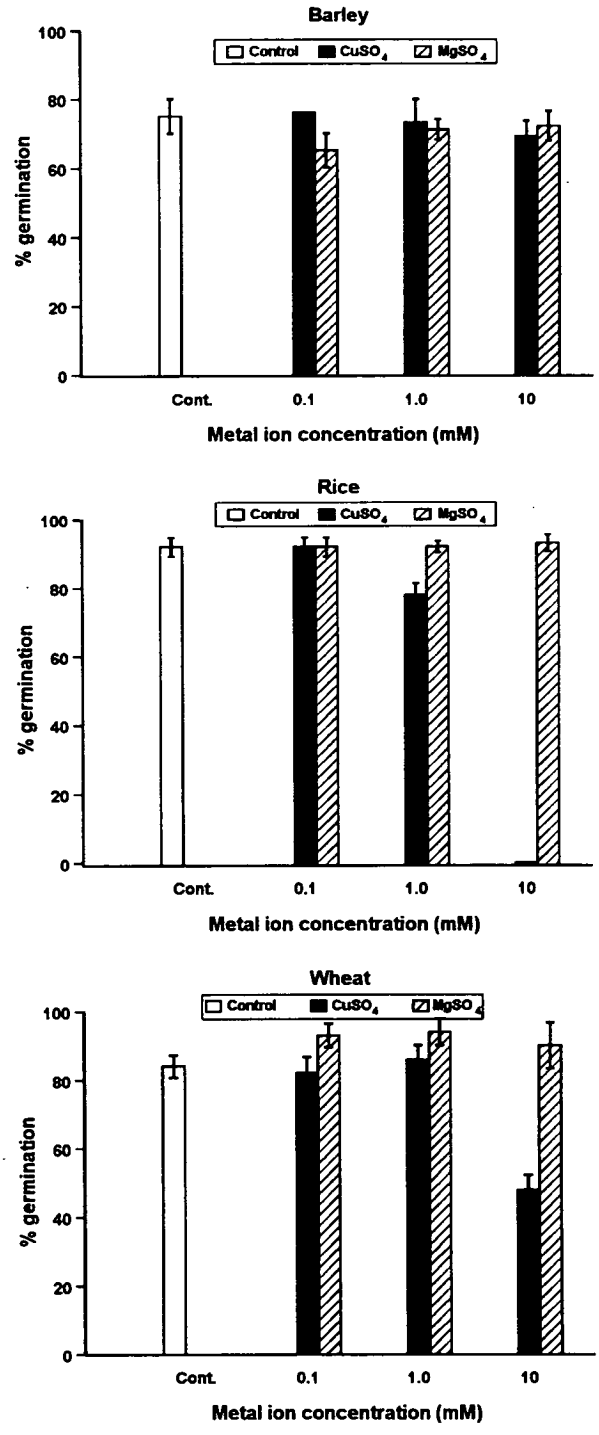


Fig. 3.1. Effect of different concentrations of metal ions on germination of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

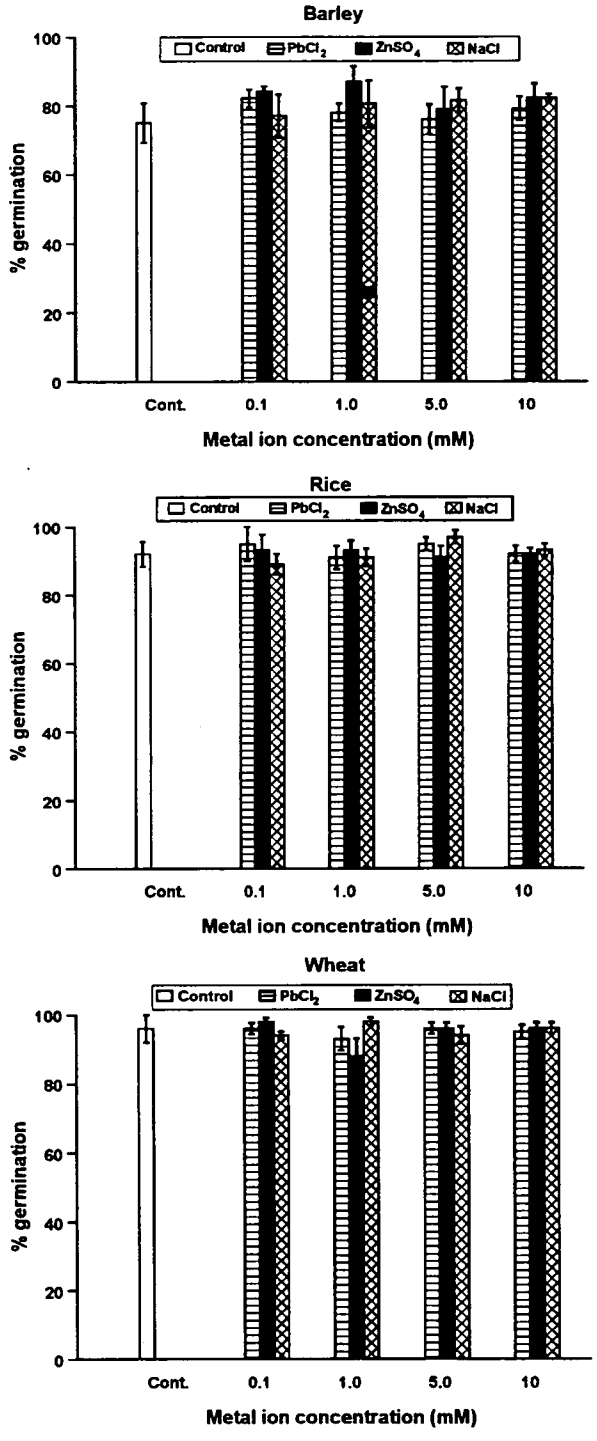


Fig. 3.2. Effect of different concentrations of metal ions on germination of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

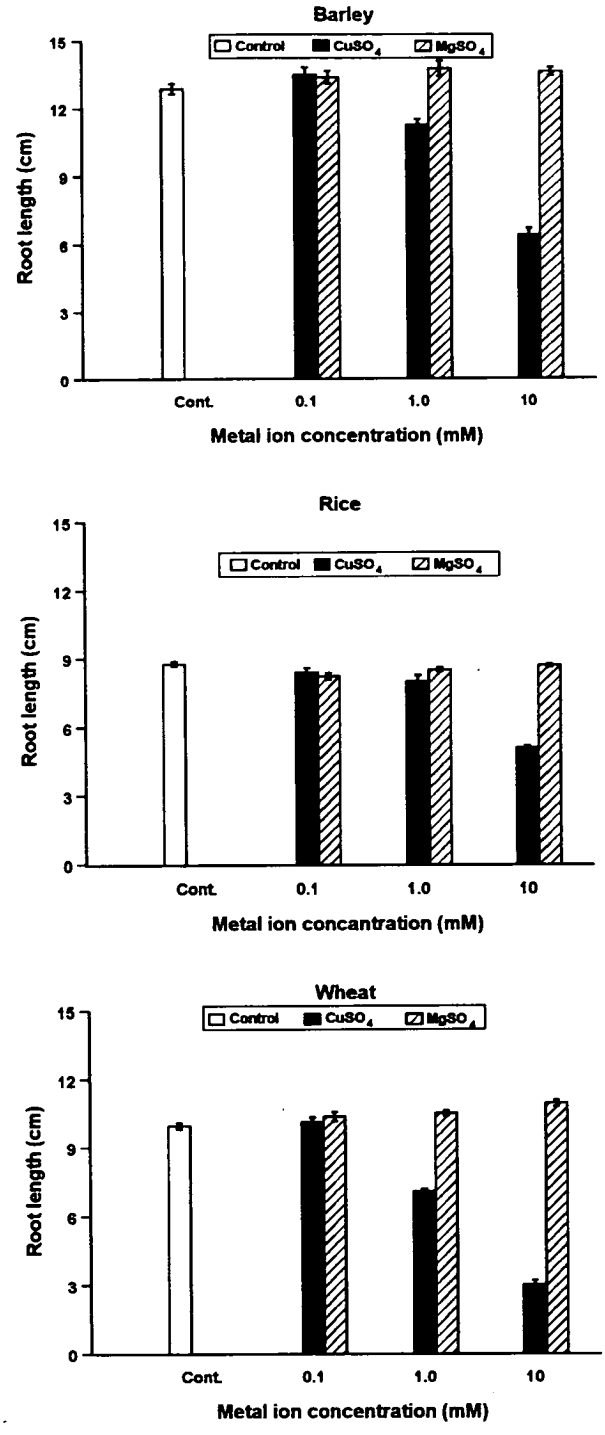


Fig. 3.3. Effect of different concentrations of metal ions on the length of the longest root of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

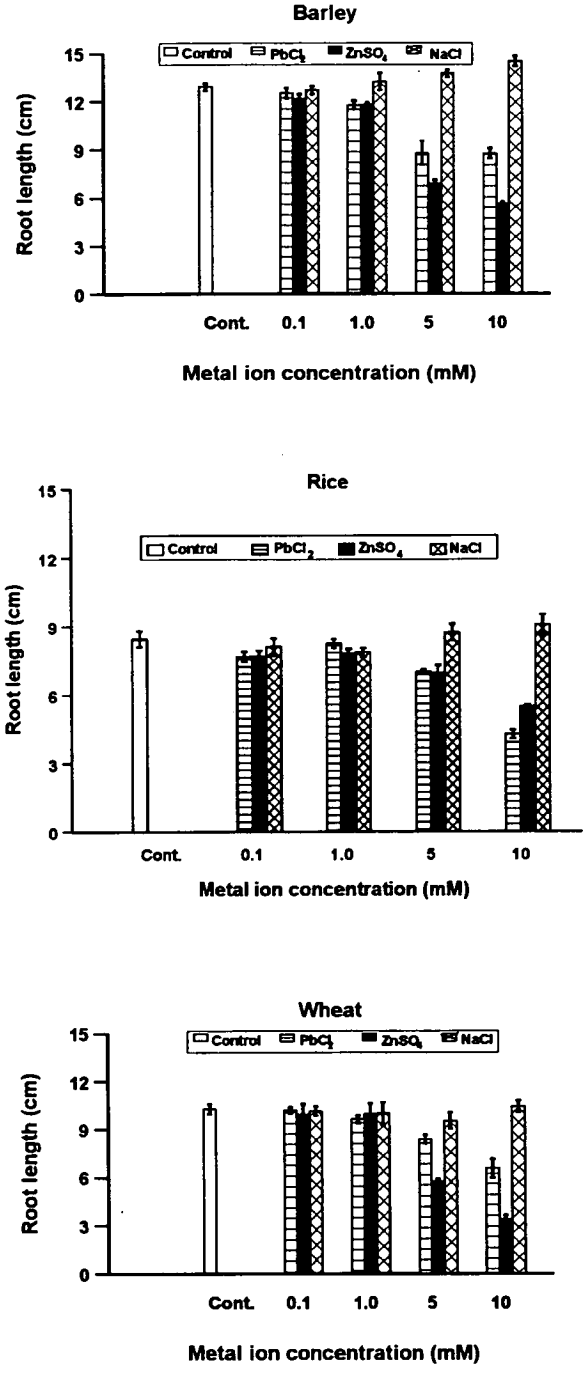


Fig. 3.4. Effect of different concentrations of metal ions on the length of the longest root of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

investigated in the three species. The percentage germination at all the concentrations of $PbCl_2$ and $ZnSO_4$ was same as that of the respective controls. The results (Fig. 3.2) show that the percentage germination in the three species in the presence of NaCl at the same range of concentrations, was same as that of the respective controls.

Root length

Barley seedlings produced the longest roots of the three species, while the shortest roots were observed in rice seedlings. Root length i.e. the mean length of the longest roots of the seedlings of the three species at 0.1 mM $CuSO_4$ was the same as that of the respective controls. However, at 1.0 mM $CuSO_4$ the root length in the seedlings of barley and wheat was 12 % and 28 % less ($p < 0.05$) than that of the respective controls, whilst the root length of the rice seedlings showed no treatment effect (Fig. 3.3). The difference between the root lengths of the seedlings in 10 mM $CuSO_4$ and the root lengths of the respective control seedlings, was greater in wheat than in barley or rice. The root length of the seedlings at all the treatment concentrations of $MgSO_4$ was similar to that of the seedlings of respective controls in the three species. The root length of the seedlings at 5 mM $PbCl_2$ was less ($p < 0.05$) than that of the respective control seedlings in the 3 species (Fig 3.4). At 5 mM $ZnSO_4$ the root lengths of the seedlings of barley and wheat were 47 % and 35 % less ($p < 0.05$) than the root lengths of the respective control seedlings, but the effect of this concentration of $ZnSO_4$ on the root length of rice seedlings was non-significant. The root length of the rice seedlings at 10 mM $ZnSO_4$ was 35 % less ($p < 0.05$) than the root length of the control seedlings and, at the same concentration, the root lengths of the other two species were 57 % less ($p < 0.05$) than the root lengths of the respective controls. In barley, the root length of the seedlings increased with increasing concentrations of NaCl and at 10 mM NaCl it was 14 % longer ($p < 0.05$) than the root length of the control seedlings. The effect of NaCl on the root length of rice and barley was non-significant.

Number of roots

In barley, which produced more roots than either of the two other species, no effect on root number was observed at any of the concentrations of $CuSO_4$ (Fig. 3.5). In wheat, root number increased by about 12 % ($p < 0.05$) over the control at 1.0 mM $CuSO_4$, but there was no difference in root number between the control and

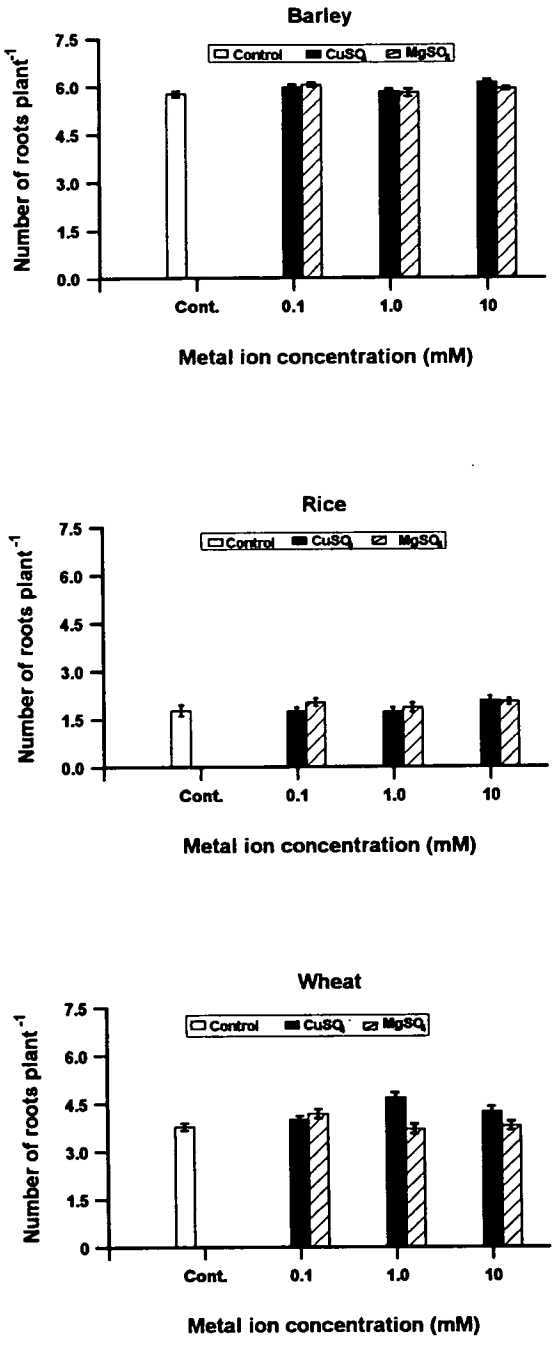


Fig. 3.5. Effect of different concentrations of metal ions on number of roots of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

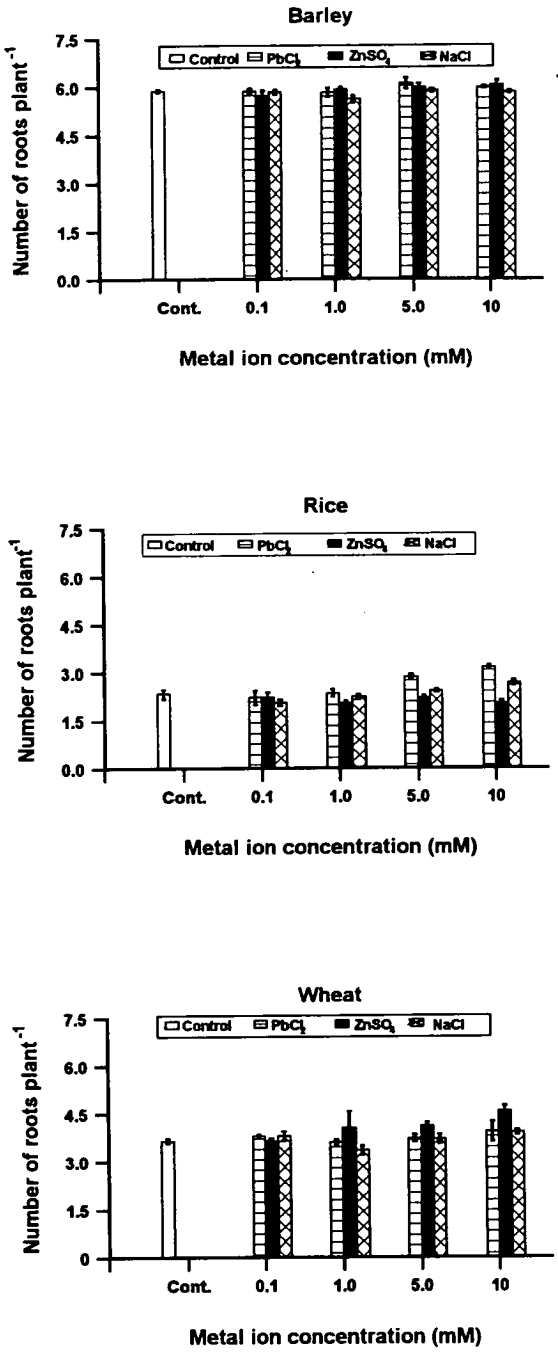


Fig. 3.6. Effect of different concentrations of metal ions on number of roots of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

10 mM $CuSO_4$. There was no effect of $MgSO_4$ on the number of roots in the three species at any of the concentrations applied. The number of roots produced in all the concentrations of $PbCl_2$ was same as that of the respective controls in barley and wheat, however, in rice it was greater at 5 mM and 10 mM $PbCl_2$ than in the control, the number at 10 mM $PbCl_2$ being 36 % more ($p < 0.05$) than that of the control (Fig.3.6). No effect of $ZnSO_4$ and NaCl was observed on the number of roots in the three species at any of the treatment concentrations.

Shoot length

In barley and rice, shoot length was not significantly different from that of the respective controls at any of the concentrations of $CuSO_4$, however, in wheat, at 10 mM $CuSO_4$ it was 47 % less ($p < 0.05$) than that of the control seedlings (Fig. 3.7). No effect of $MgSO_4$ was observed on the shoot length of any of the three species at any of the concentrations used. Compared to the respective controls, the shoot length of barley increased with increasing concentrations of $PbCl_2$, whilst the shoot length of rice and wheat showed a slight inhibition at higher concentrations (Fig. 3.8). The effect of $ZnSO_4$ on the shoot length in barley and wheat seedlings followed a pattern similar to that which was observed for $PbCl_2$. In contrast, the shoot lengths in rice seedlings at 5 mM and 10 mM $ZnSO_4$ were 18 % less ($p < 0.05$) than those of the control seedlings. The effect of NaCl on shoot length of all three species was non-significant.

Root:shoot ratio

The root:shoot ratio in the three species at 0.1 mM $CuSO_4$ was the same as that of the respective controls. At 1.0 mM $CuSO_4$, the root:shoot ratio in barley and wheat was 21 % and 30 % less ($p < 0.05$) than that of the respective controls, respectively. At this concentration there was no effect on the root:shoot ratio in rice seedlings (Fig. 3.9). However, root:shoot ratio of rice seedlings grown at 10 mM $CuSO_4$ was 32 % less ($p < 0.05$) than that of the control seedlings. The root:shoot ratio was unchanged in response to different concentrations of $MgSO_4$ in all the three species. In barley, the root:shoot ratio at 5 mM $PbCl_2$ was 41 % less ($p < 0.05$) than that of the control, while in rice there was a non-significant difference between the seedlings grown at 5 mM and the control seedlings, and a significant ($p < 0.05$) difference between the seedlings grown at 10 mM and the control seedlings. At 5 mM $ZnSO_4$ the root:shoot ratio in barley, was 57 % less ($p < 0.05$) than that of the control and was unaffected in case of rice, whereas in wheat at 5 mM $ZnSO_4$ it was

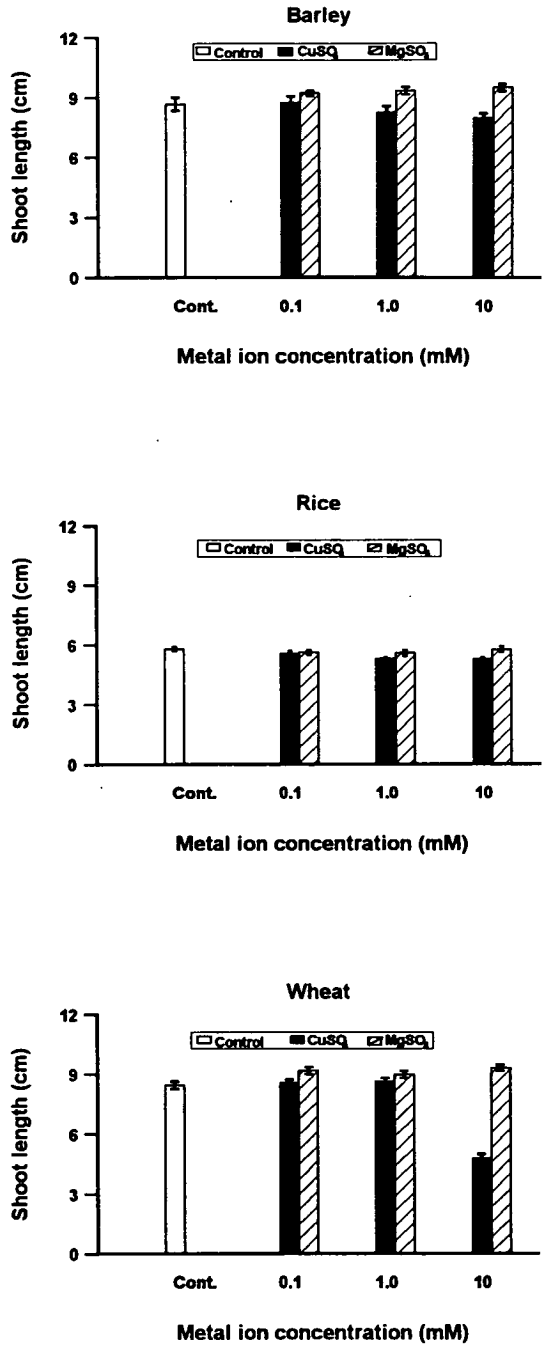


Fig. 3.7. Effect of different concentrations of metal ions on shoot length of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

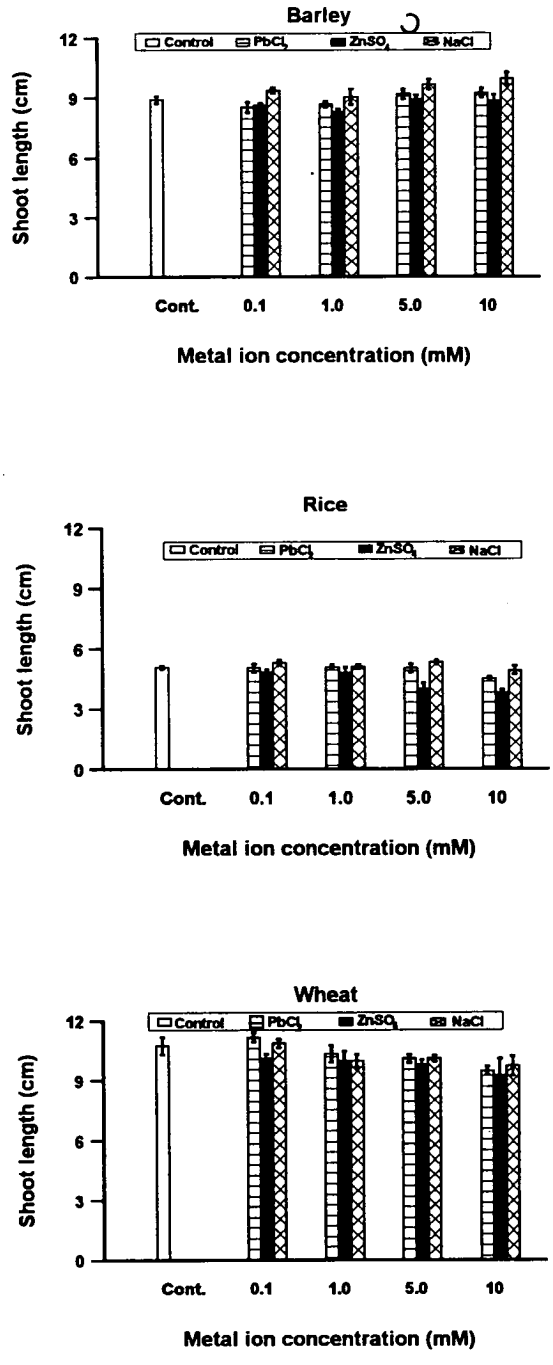


Fig. 3.8. Effect of different concentrations of metal ions on shoot length of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

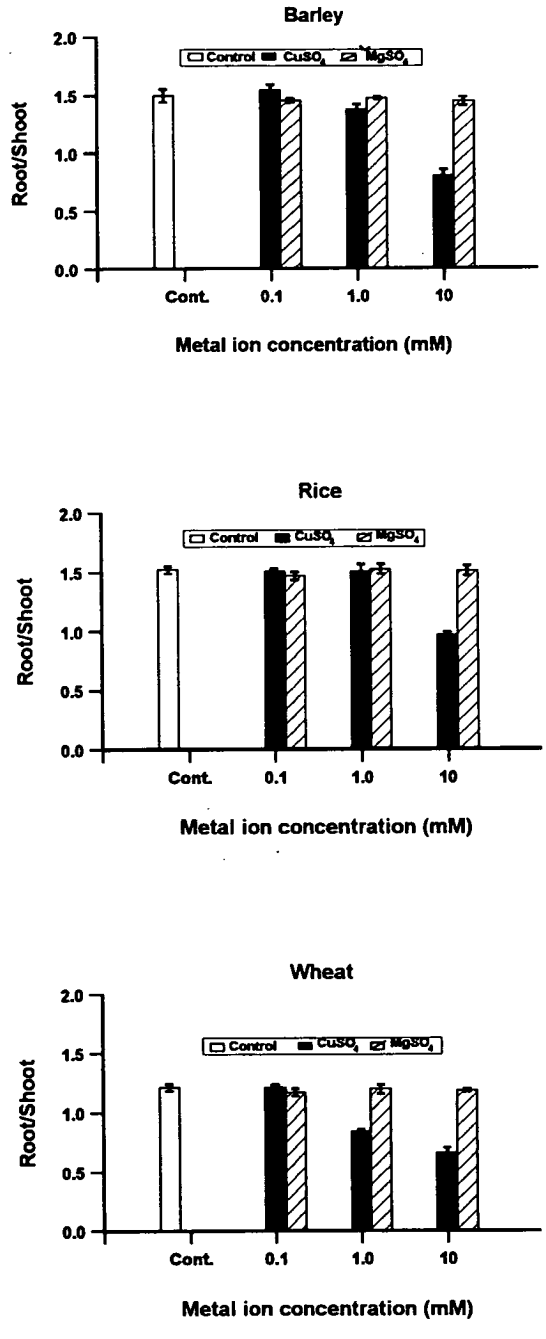


Fig. 3.9. Effect of different concentrations of metal ions on root/shoot ratio of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

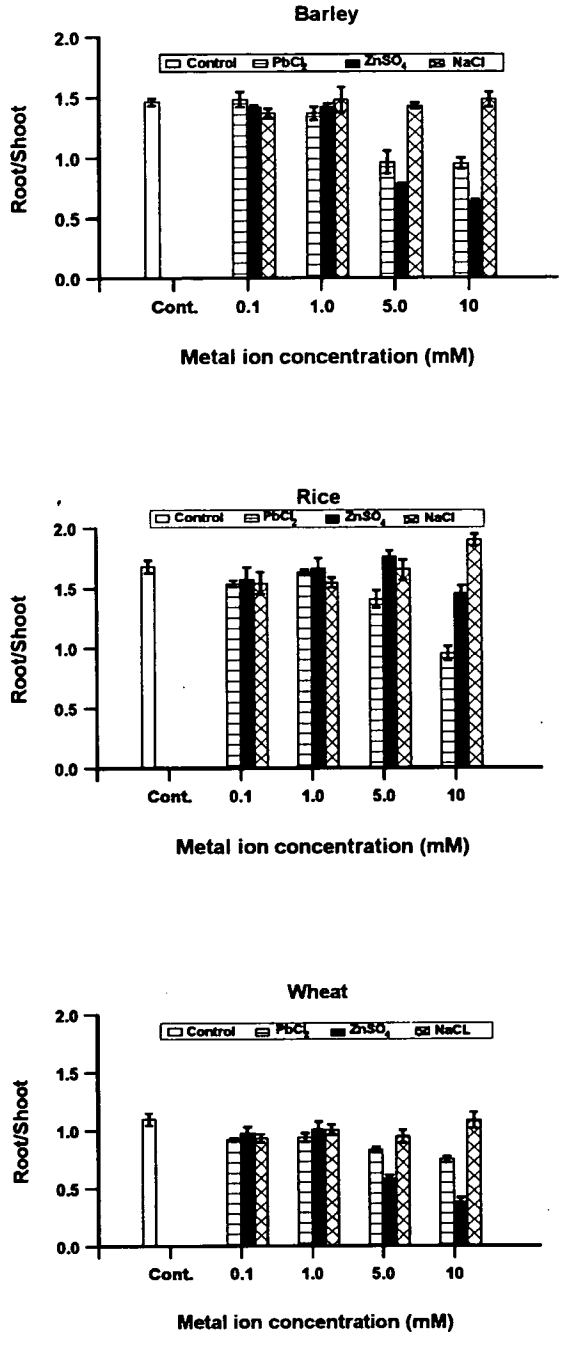


Fig. 3.10. Effect of different concentrations of metal ions on root/shoot ratio of barley cv. Triumph, rice cv. NIAB 6 and wheat cv. Tonic. Error bar ± SEM, n=4.

Table 3.1 Summary of the effects of copper, lead and zinc on germination and seedling growth of barley, rice and wheat.

Metal ions	Concen-trations	% germination			Root length			Number of roots			Shoot length			Root:Shoot ratio		
		Barley	Rice	Wheat	Barley	Rice	Wheat	Barley	Rice	Wheat	Barley	Rice	Wheat	Barley	Rice	Wheat
Cu																
	0.1 mM	0	0	-	+	-	+	+	-	+	-	-	+	+	0	0
	1.0 mM	-	-	+	-*	-	-*	0	-	+*	-	-	+	-	0	-*
	10 mM	-	n	-*	-*	-*	-*	+	+	+	-	-	-*	-*	-*	-*
Pb																
	0.1 mM	+	+	0	-	-	-	0	-	+	-	0	+	+	-	-*
	1.0 mM	+	-	0	-	-	-	-	0	-	-	0	-	-	+	-
	5.0 mM	+	+	0	-*	-*	-*	+	+	+	+	0	-	-*	-	-*
	10 mM	+	0	0	-*	-*	-*	+	+*	+	+	-*	-	-*	-*	-*
Zn																
	0.1 mM	+	0	+	-	-	-	-	-	0	-	-	-	-	-	-
	1.0 mM	+	0	-	-	-	-	+	-	+	-	-	-	-	0	-
	5.0 mM	+	0	0	-*	-	-*	+	-	+	+	-*	-	-*	+	-*
	10 mM	+	0	0	-*	-*	-*	+	-	+*	+	-*	-	-*	-	-*

0 = no effect

+ = increase

- = decrease

* = significant at 95 % confidence interval

n = no germination

29 % less ($p < 0.05$) than that of the control. With NaCl, no change in root:shoot ratio was observed in any of the three species.

At 1.0 mM and 10 mM $CuSO_4$, and 5 mM and 10 mM $PbCl_2$ and $ZnSO_4$ the root tips of the seedlings of all the species were brown to dark brown in colour and roots were without root hairs. The roots were stunted, thick, curled and brittle with small lateral branches, whereas the roots of the seedlings grown in control or lower metal ion treatments were white and long, with profuse root hairs and long lateral branches.

Table 3.1 shows a summary of the effects of the metal ions on the three species. A very small effect was observed in percentage germination, whereas root length showed most of the significant effects of different treatment concentrations in the three species.

3.4. Discussion

According to the definition of Bewley and Black (1994) germination begins with water uptake by the seed and ends with the start of elongation by the embryonic axis, usually the radicle; therefore on emergence of the radicle, the seed is considered to be germinated. Germination tests are generally carried out by spreading seeds on solid media (filter paper or sand) soaked with the treatment solutions, incubating in specific conditions of temperature and humidity and counting the number of seeds which have germinated after a given period of time (Gupta and Mukherji 1977; Leblová *et al.* 1986; Ibrahim *et al.* 1989; Hasnain *et al.* 1993; Kumar *et al.* 1993). However, none of these workers has mentioned the criterion which was used to classify the seeds as germinated. In order to have a practical and easily recognisable criterion, Kaur and Duffus (1989), studying the effect of fluoride ions on germination of wheat, rice and barley using soaked filter paper, considered that seeds which had a minimum of 5 mm radicle length 96 h after the start of imbibition were germinated. In the presently reported work, the method of Kaur and Duffus (1989) was used to investigate the effect of copper, lead and zinc ions on the germination of barley, rice and wheat. In order to discover whether or not the observed effects on germination were due to cations or anions, germination tests were carried out using $MgSO_4$ and NaCl at the same concentrations and under the same conditions. Since no inhibitory effect of $MgSO_4$ and NaCl was observed, even in rice seeds, it may be concluded that

the inhibition in germination caused by $CuSO_4$ was a consequence of copper ion toxicity, at the concentrations used.

Germination was inhibited by $CuSO_4$ only at the highest concentration i.e. 10 mM, and lead and zinc ions had no effect on the process of germination at any of the concentrations used. Previous work (Burcky and Winner 1979; Pahlsson 1989) has also reported that copper is a more toxic metal ion than zinc and lead in the germination process. Of the three species, rice seeds failed to germinate at 10 mM $CuSO_4$,whereas, at the same concentration, germination inhibition was 47 % in wheat and zero in barley. Gupta and Mukherji (1977) reported a complete inhibition of germination in rice at 100 mM copper. Present findings showed that at a copper concentration of 10 mM rice seeds showed a slight protrusion of the radicle but this was not classified as germinated according to the criterion followed. Complete inhibtion of germination may be caused by concentrations of copper ions lower than 100 mM but in the work reported, the metal ion concentrations used in multiples of ten (Mukherji and Gupta 1972; Gupta and Mukherji 1977; Leblová *et al.* 1986) and the effects of concentrations between 10 and 100 mM were not determined. Inhibition of seed germination by copper ions may be due to interference with some important enzymes involved in the process. It is difficult to investigate the physiological effects of metal ions on the process of germination because germination is considered to be complete when the radicle has emerged. Processes occurring in the young seedling, such as mobilisation of the major storage reserves, are not considered as part of germination, rather classed as post-germination events (Bewley and Black 1994).

Investigations on the effect of metal ions on seedling growth have been carried out in sand (Burcky and Winner 1979; Chrenekova 1981). Sand culture provides a solid medium and approximates more closely than water culture to soil conditions. However, it is very difficult to avoid contamination due to the presence of micronutrients in sand. In addition, removal of seedlings with a complete root system intact is also difficult. Gupta and Mukherji (1977), Leblova *et al.* (1986), Kumar *et al.* (1993), and Hasnain *et al.* (1993) while studying the effect of metal ions on seedlings growth of rice, maize, sesamum and wheat, respectively, used filter paper soaked with treatment solutions. However, Perry (1977) recommended the use of the rolled paper towel test as a better method for the assessment of seedling growth in cereals. Kaur and Duffus (1989) used a modification of Perry's method to study the effect of fluoride ions on the seedling growth of rice, wheat and barley. After pasting

seeds on the sheets of germination papers they moistened the sheets with the treatment solutions and loosely rolled them along the lower edge around a 100 cm^3 conical flask to form a tube of about 40 mm diameter. These tubes of paper towel were then put in trays of the treatment solutions in incubators for 7 d, the trays being topped up daily with the respective treatment solutions. In the present study, a further development of Perry's method was used. Here the rolled sheets of paper towel, on which seeds had been pasted, were placed inside specimen tubes containing 100 ml of the treatment solution and were incubated in a controlled environment cabinet. The solution levels in the specimen tubes were maintained by adding ultra-pure water only. This is a more convenient method to study the effect of each metal ion on seedling growth in different species in a single large experiment in uniform conditions.

When the effect of the metal ions on seedling growth parameters was monitored, a significant inhibition in shoot length of the seedlings was observed only in rice at 5 mM lead and 10 mM zinc, and in wheat at 10 mM copper. No inhibition was observed in the shoot length of barley. The growth of the longest roots of the seedlings was more severely inhibited than the growth of the shoot. More inhibition of root elongation than of shoot elongation has been reported in seedlings of wheat due to copper ions (Ibrahim *et al.* 1989) and lead ions (Hasnain *et al.* 1993), and in maize due to zinc, copper and lead ions (Leblova *et al.* 1986) . Of the three toxic metal ions tested in the present investigation, copper ions appear to be more toxic than zinc and lead, as significant inhibition of root elongation in barley and wheat seedlings was observed at 1.0 mM copper. At this concentration, rice seedlings also showed inhibition of root elongation in 7 d old seedlings, but the effect was not significant. Gupta and Mukherji (1977) reported that a 1.0 mM solution of copper ions was toxic enough to inhibit significantly root elongation in rice seedlings grown on soaked filter paper for 5 d. Lead was more toxic than zinc but less toxic than copper. The three species showed a significant inhibition in root length of the seedlings at a 5 mM concentration of lead ions. These results are similar to the findings of Hasnain *et al.* (1993) who reported a significant inhibition in root length of seedlings of wheat grown for 7 d on filter paper soaked with 5 mM lead acetate. In rice seedlings, significant inhibition in root length was observed at a 10 mM concentration of zinc ions. Of the three species, barley and wheat were more sensitive to copper and zinc in terms of the inhibition of root elongation, while rice was sensitive to these metal ions only at higher concentrations. All species showed equal sensitivity to lead. Previously, no comparison between these three species with

respect to the effect of copper, zinc and lead ions on the root growth of the seedlings had been reported.

Changes observed in the structure and morphology of the roots due to copper, lead and zinc ions were similar for all the species and confirm those results reported by Pahlsson (1989). Apparently root browning is caused by enhanced suberization and the deposition of polyphenols and may also occur as a result of the toxicity of cadmium, copper, manganese, cobalt and nickel (Patterson and Olson 1983; Hagemeyer *et al.* 1986). The concentrations of metal ions which caused structural changes also inhibited growth of root hairs. Hasnain *et al.* (1993) and Kumar *et al.* (1993), studying the effect of lead ions on seedlings of wheat and sesame respectively, also found no root hairs on the roots of the seedlings grown on paper soaked in a solution of lead ions. Change in the root:shoot ratio of the seedlings was either due to greater inhibtion of root length than the inhibtion in shoot length or due to inhibition of root length only.

A major function of the roots of seedlings is to transfer in a regular manner, mineral nutrients and water from the soil solution to the shoot, thus enabling the successful establishment of the seedlings. Metal ion-induced changes in the structure of roots such as absence of root hairs, stunted root growth, thickening or browning, may cause restriction in absorption of water and nutrients and thereby a decrease in seedling growth.

Though rice appeared more sensitive to metal ions at germination than barley and wheat, it maintained a better root:shoot ratio than the other two species during seedling growth in the presence of toxic metal ions. Copper ions produced a stronger inhibitory effect on seed germination and seedling growth than did zinc and lead, and in the presence of each of the three ions at toxic concentrations root elongation was inhibited more than shoot elongation. There was no relationship between the concentrations of metal ions which inhibited the process of germination and the concentrations which were toxic to seedling growth. However, the concentrations of metal ions which caused toxic effects on seedlings were quite high compared to the concentrations reported in studies which have been carried out using solution culture experiments (Craig 1978). Craig (1978) found 0.3 μM copper, 11.2 μM lead and 8.37 μM zinc toxic enough to cause 50 % inhibition in root growth of *Zea mays*. Filter papers used for germination tests and paper towels used for seedling growth estimations are made of cellulose which appears to have a very strong

adsorption capacity for metal ions. It was found that within 12 h the paper towels used for seedling growth tests had adsorbed about 50 % of the copper ions from a solution of copper sulphate (Table 2.1.). This may give rise to considerable variation in treatment concentrations when the effect of metal ions is under investigation. Repeated application of the metal ion solution by some workers (Kaur and Duffus 1989; Hasnain *et al.* 1993) in order to keep the paper wet has presumably resulted in many-fold application of metal ions. The results of experiments carried out using paper as the supporting medium are valid in themselves if they are carried out carefully using a standard regime, but they can not be compared with the results obtained by other workers using the same method or with the results of experiments using liquid culture. The results obtained in the present experiments were reproducible and the errors were small, but, because of the adsorption of ions by paper, this method could not be used to determine the exact concentration of metal ions which causes a toxic effect.

4. ASSESSMENT OF COPPER ION-INDUCED CHANGES IN SEEDLING GROWTH OF RICE (*Oryza sativa* L.), THEIR RELATIONSHIP TO THE pH OF THE NUTRIENT SOLUTION, AND TO POTASSIUM ION LEAKAGE AND ROOT LIPID PEROXIDATION.

4.1. Introduction

Above a certain concentration, metal ions are toxic to many organisms, including higher plants (Verkleij and Schat 1990). Many investigations have been carried out on the toxic effects of copper ions on the physiological and biochemical aspects of plant growth and development. Sandman and Bögar (1980) and Stiborova *et al.* (1986) studied the effect of copper ions on the physiology and biochemistry of enzymes involved in metabolism and photosynthesis. Lidon and Henriques (1991, 1992 a,b) reported the effects on seedling growth, photosynthesis, copper uptake kinetics and their influence on uptake and utilization of other nutrients. All these aspects of plant growth were inhibited to some extent depending upon the concentration of copper ions. It is extremely difficult to identify exactly the lowest copper ion concentration in the root medium at which the symptoms of metal ion toxicity appear, as there is variation between species and between ecotypes of individual species, and also because, in metal ion studies, plants are generally subjected to doses which definitely produce toxic effects. Lidon and Henriques (1992 b) have recently reported a significant inhibition in the root elongation of 30 d old rice seedlings grown in 20 μM copper ion concentration. However, in the same study, the effects on other seedling growth parameters were observed at relatively higher copper concentrations.

Roots are the parts of the plant most directly exposed to the detrimental effect of toxic metal ions in the environment, except in the case of aerial metal deposition (Ernst 1980). Roots are therefore the initial site of metal ion toxic effects. As a consequence, root growth is the most widely used parameter for the assessment of metal ion toxicity. The usefulness of root growth as a parameter in the assessment of metal ion toxicity probably relies on the fact that the metal ion-imposed root growth reduction is due to a direct effect of the metal on the root itself (Ernst *et al.* 1992). However, there is no reason why the growth of any part of the plant or of the whole plant should not be used in assessments (Magnavaca *et al.* 1987).

pH

It is recognised that low pH greatly affects the physiology of root systems resulting in stunted, highly branched and coarse roots (Larcher 1980). In most of the studies on the toxic effect of copper ions on root growth, seedlings have been grown in nutrient solutions supplemented with elevated concentrations of copper ions at pH 5.5 (De Vos *et al.* 1989; Thornton and Macklon 1989; Lidon and Henriques 1991, 1992 a,b). It has been shown that the toxic effects on seedlings caused by aluminium ions increase as the pH of the solution is lowered, perhaps due to an increase in the availability of aluminium ions at low pH (Marschner 1991) However, certain species such as European beech, are known to be much more sensitive to low pH than to the effect of aluminium ions (Murah and Ulrich 1988). In view of this effect and the fact that soil pH varies considerably under natural conditions it was decided to investigate the effect of pH of the solutions on the toxic effects of copper ions during seedling growth.

K^+ leakage and lipid peroxidation

A known toxic effect of copper is the alteration of plasmamembrane permeability leading to leakage of solutes including ions such as K^+ and other solutes (Wainwright and Woolhouse 1977; Ohsumi *et al.* 1988). De Vos *et al* (1989) incubated roots of the seedlings of *Silene cucubalus* in toxic concentrations of copper ions. After a certain time period, the K^+ ion concentration in the external solution was found to have increased relative to the control. On staining the same roots with trypan blue, after the incubation period, they observed that roots from toxic concentrations of copper ions were deeply stained, indicating damage to the plasmamembrane of the root cells. They suggested that increase in K^+ ion concentration is the result of leakiness in the plasmamembrane of the root cells caused by the copper ions. It has been reported that the permeability of membranes depends on the degree of lipid peroxidation (Dhindsa *et al.* 1981). Copper-induced lipid peroxidation has been demonstrated in isolated chloroplasts (Sandmann and Böger 1980). De Vos *et al* (1989) observed an increase in lipid peroxidation products in the roots subjected to toxic concentrations of copper ions and concluded that lipid peroxidation of the plasmamembrane by copper ions could be one of the primary effects of copper ions on plasmamembrane integrity, resulting in leakage of K^+. In all the previous work done on this topic *Silene cucubalus*, *Silene vulgaris* or *Mimulus guttatus* have been used and there is no published information on the effect of copper ions on the leakage of K^+ or on lipid peroxidation in the roots of rice seedlings.

Therefore the following series of experiments was carried out with three main objectives. The first was, to determine the minimum concentration of copper ion which gives rise to an observable toxic effect in rice seedlings. The second objective was, to determine the influence of pH of the nutrient solution on the copper ion-induced toxicity in the rice seedlings. And the third objective was to investigate the relationship between the lowest copper ion concentration which inhibits root growth-induced K^+ leakage and the increase in lipid peroxidation in the roots of the rice seedlings and to determine whether this effect of copper ions is time-dependent or dose-dependent.

4.2. Determination of the lowest copper ion concentration which will induce changes in seedling growth. (Experiment I)

4.2.1. Experimental Procedure

Grains of rice (*Oryza sativa* L.) cvs. Basmati 370 and NIAB 6 were obtained from the Saline Agricultural Research Cell, Department of Soil Science, University of Agriculture, Faisalabad, Pakistan. Basmati 370 is a tall-statured (> 90 cm) cultivar. It has low yield potential, but the grains are long and of fine quality, with a very pleasant aroma. In contrast, NIAB 6 is a short-statured (40 cm) cultivar with a high yield potential. It has coarse grains with very little aroma. These two cultivars differ in their susceptibility to a number of stress factors including insect pests, disease and salinity. Basmati 370 is one of the most salinity-sensitive cultivars, whereas NIAB 6 is a salinity-tolerant cultivar. There is no background information about the metal ion tolerance of these two cultivars.

The grains were stuck to black polyethylene sheets and grown under the conditions described in Section 2.2.1. Fresh Yoshida nutrient solution was prepared and left overnight for stabilisation. A stock solution of 1 mM copper sulphate was prepared (using $CuSO_4.5H_2O$) and added to the nutrient solution to produce treatment levels of 1(control), 4, 8 and 16 μM copper ions. Each treatment comprised eight specimen tubes, and each treatment was replicated three times i.e. a total of 192 specimen tubes. The experiment was set up in two growth cabinets. Each contained two of the four treatments. The treatment solutions were replaced on alternate days. Two sets of specimen tubes were used to facilitate a quick change of the solutions. After use, each set was washed thoroughly and then rinsed with distilled water to reduce the chance of algal growth.

When the seedlings were 20 d old the experiment was harvested. The seedlings which had morphological abnormalities were removed and lengths of the shoot and of the longest root of 100 seedlings were measured for each replication. The seedlings were washed thoroughly in distilled water and blotted dry, separated into roots and shoots at the embryonic axis for fresh and dry weight and chemical analyses as described in Section 2.4.

4.2.2. Results

The mean length of the longest roots, of 20 d old seedlings of two cultivars of rice grown in solutions of different concentrations of copper ions is presented in Fig. 4.1. In NIAB 6, there were no differences between the root lengths of the seedlings grown in the control, 4 and 8 μM copper ion solution but the root length of the seedlings grown in the 16 μM copper ions solution was 28 % less ($p < 0.05$) than the root length of the seedlings grown in the control solution. The seedlings of the cultivar NIAB 6, had a longer root length than the seedlings of the Basmati 370, when grown in the control, 4 and 8 μM copper ion solutions. However, when the seedlings were grown in the 16 μM copper ion solution, the root length was same in both the cultivars. The root lengths of the Basmati 370 seedlings grown in the 4 μM and 8 μM copper ion solutions were similar to the root lengths of the seedlings grown in the control solution. When the seedlings of the Basmati 370 were grown in the 16 μM copper ion solution the root length was 23 % less ($p < 0.05$) than the root length of the seedlings grown in the control solution.

The shoot lengths i.e. the mean length of the shoots, of both Basmati 370 and NIAB 6 seedlings were the same when the seedlings were grown in the control and at all concentrations of copper. However, the shoot length of the NIAB 6 seedlings was 17 % ($p < 0.05$) smaller than the shoot length of the Basmati 370 seedlings when grown in the control and at all concentrations of copper.

The effect of different concentrations of copper ions on root and shoot fresh weight plant^{-1} of 20 d old seedlings of the rice cultivars is presented in Fig. 4.2. Despite of some inhibition in root elongation at 8 and 16 μM copper, in NIAB 6 the root fresh weight of the seedlings grown in these treatments was statistically same as that of the control under this experimental condition. The root fresh weight of the seedlings of Basmati 370 was the same as when grown in the control

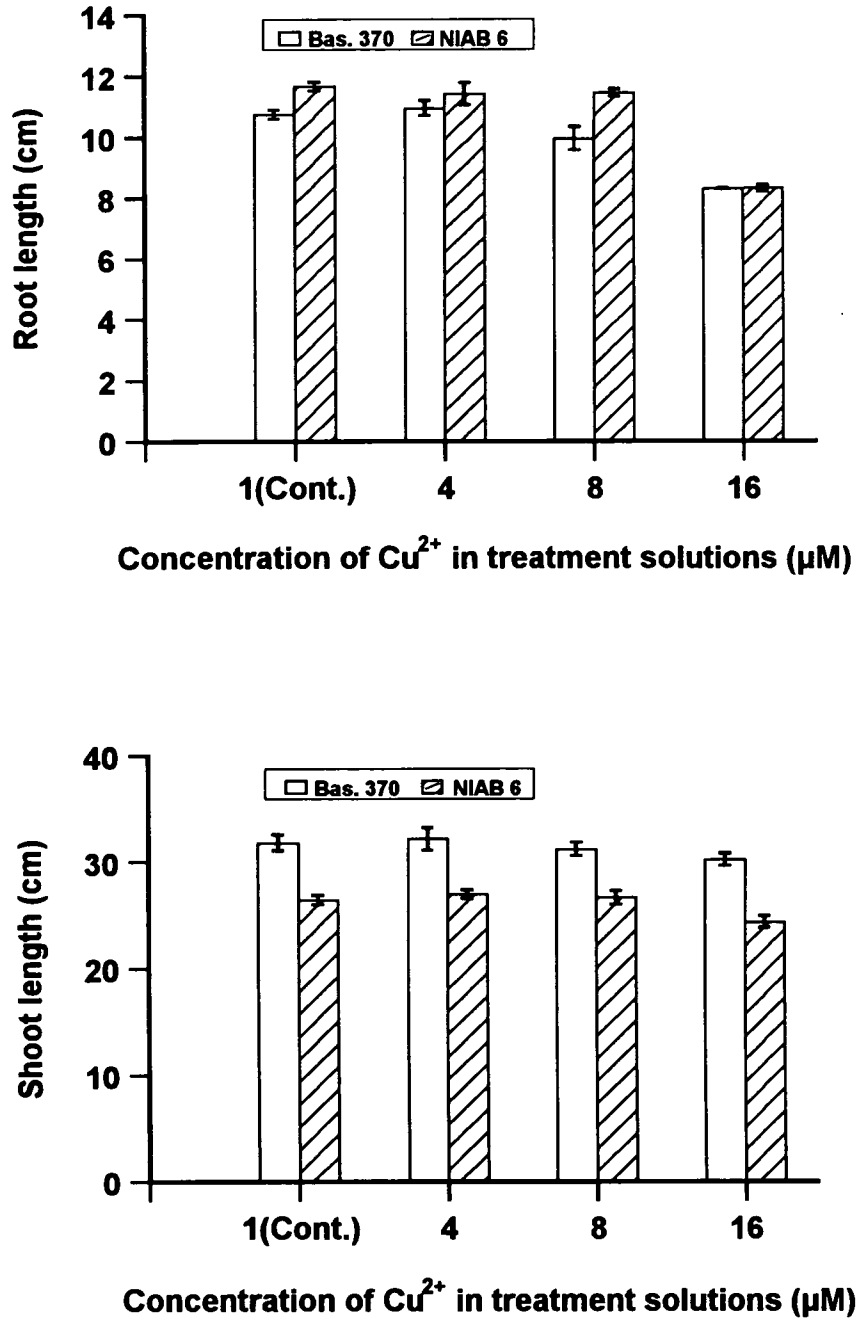


Fig. 4.1. Effect of different concentrations of copper ions on the length of the longest root, and of the shoot of 20 d seedlings of two cultivars of rice (Basmati 370 and NIAB 6). Error bar ± SEM, n=3.

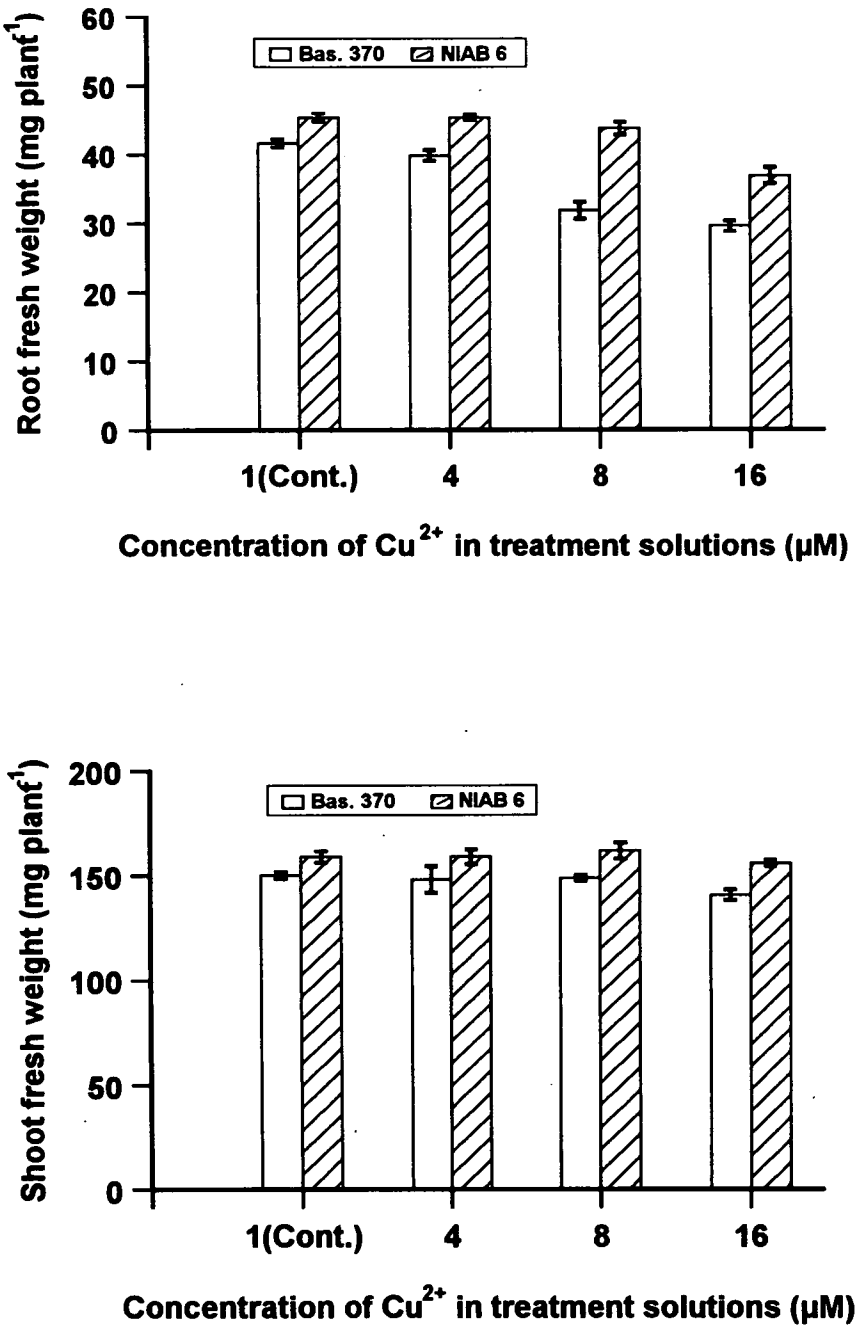
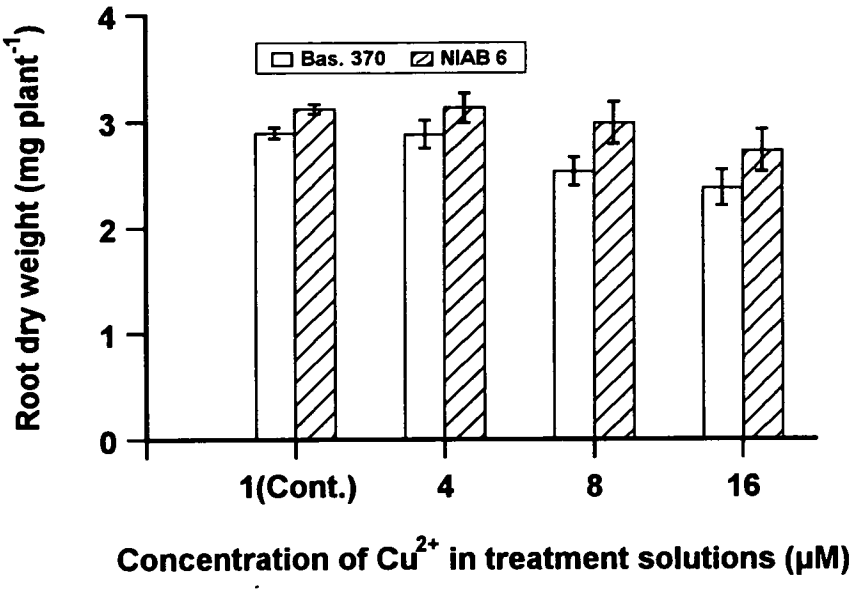


Fig. 4.2. Effect of different concentrations of copper ions on root and shoot fresh weight of 20 d seedlings of two cultivars of rice. Error bar ± SEM, n=3.

and 4 μM copper ion solution. However the root fresh weight of the seedlings grown in the 16 μM copper ion solution was 29 % less ($p < 0.05$) than the root fresh weight of the seedlings grown in the control solution. The root fresh weight of the NIAB 6 seedlings was 16 % ($p < 0.05$) higher than the root fresh weight of the seedlings of Basmati 370 grown in the control solution and at all concentrations of copper. No effect of copper ion treatments on the shoot fresh weight was observed in either of the cultivars, but the NIAB 6 seedlings had a higher shoot fresh weight than the shoot fresh weight of the seedlings of Basmati 370 in the control and in all the copper ion solutions.

Root and shoot dry weights of the seedlings were measured and were expressed in mg plant^{-1} (Fig. 4.3). Root dry weight in both the cultivars followed a pattern similar to that observed for root fresh weight. The root dry weight of the seedlings of NIAB 6 grown in the control solution was the same as the root dry weight of the seedlings grown in the 4, 8 and 16 μM copper. Similarly the root dry matter of the Basmati 370 seedlings was the same as in the control and in all copper concentrations. However, in contrast to the root fresh weight of the cultivars, the root dry weight of the seedlings of the cultivars was not significantly different. Analysis of the shoot dry weight of the cultivars showed that there was no inhibitory effect of copper ions even when the seedlings were grown in 16 μM copper ion solution. However, shoot dry weights of the seedlings of both the cultivars grown in the 4 μM copper ion solution were more than those of the seedlings grown in the control solutions. The shoot dry weight of NIAB 6 seedlings was greater than that of Basmati 370 seedlings in the control and in all the copper ion treatments, but the differences were non-significant.

The forms of copper present in complete Yoshida nutrient solution at pH 5.5 were calculated by the computer simulation programme GEOCHEM-PC (Parker *et al.* 1994). No insoluble copper complex was formed in any of the treatment combinations. The percentages of added copper ions in the treatment solutions which were present as free metal ions or complexd with EDTA are presented in Fig. 4.4. The results showed that for all the treatments, more than 99 per cent of copper added to the nutrient solution, was present complexed with EDTA and a very small fraction was available as free copper ions.



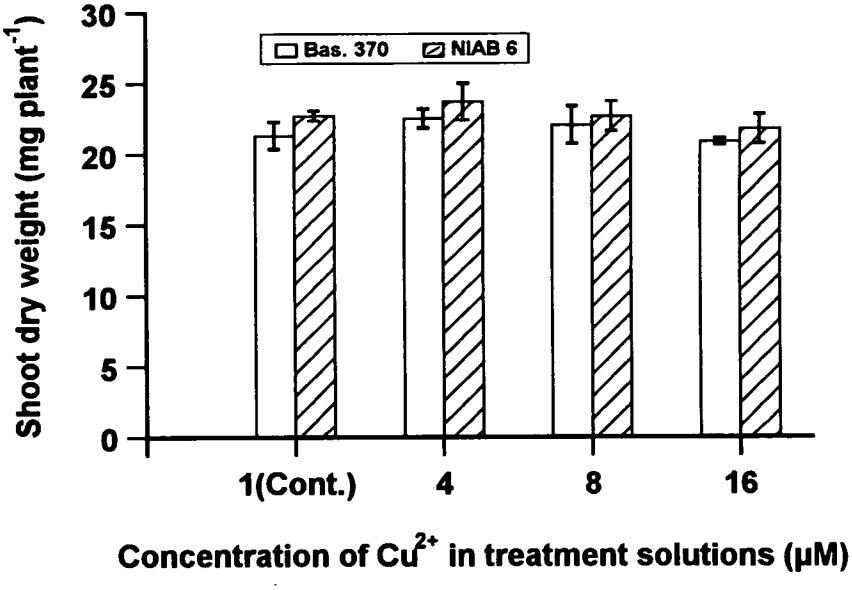


Fig. 4.3. Effect of different concentrations of copper ions on root and shoot dry weight of 20 d seedlings of two cultivars of rice. Error bar ± SEM, n=3.

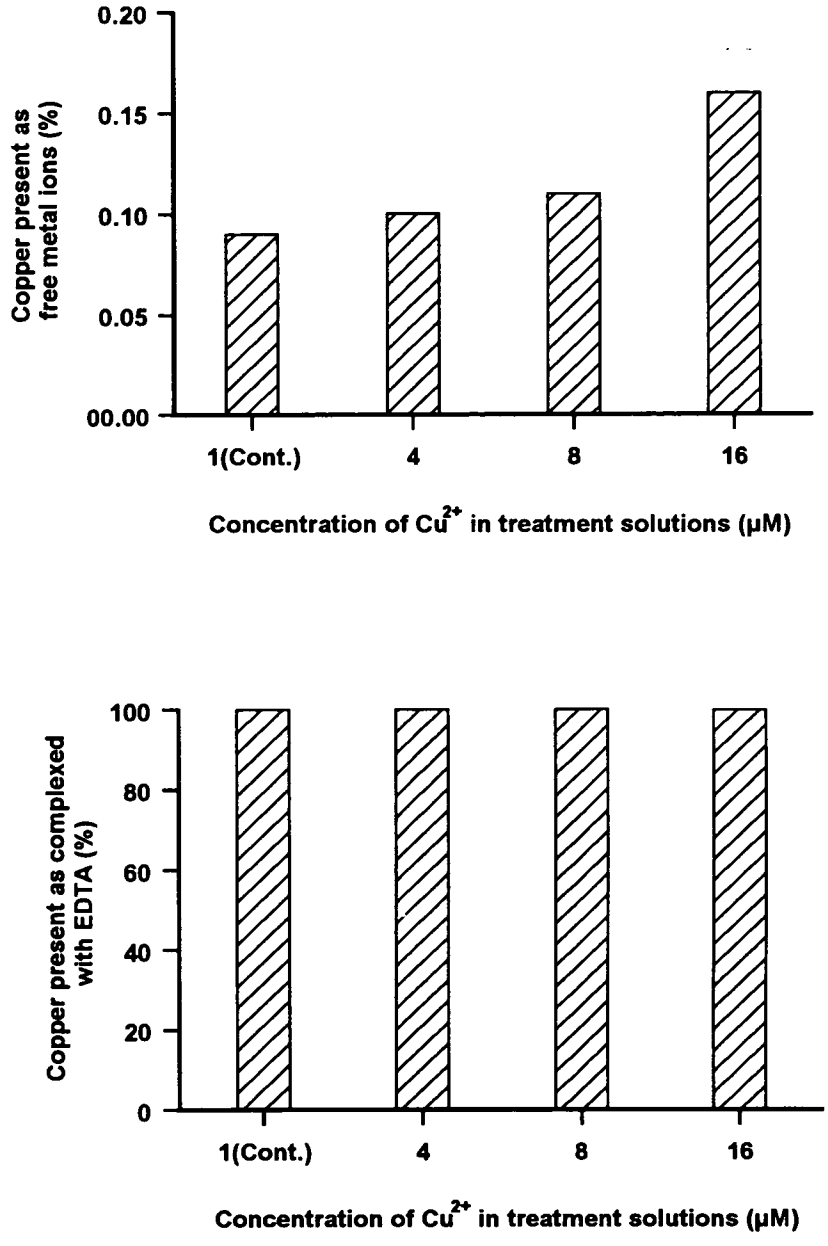


Fig. 4.4. Percentage of added copper ions present in ionic form and complexed with EDTA in complete Yoshida nutrient solution at pH 5.5 as predicted by GEOCHEM-PC.

There was a significant ($p < 0.05$) effect of copper ion treatment on the accumulation of copper in the root tissues of both of the cultivars (Fig. 4.5). A parallel and consistent increase in the amount of copper accumulated in root tissues of both of the cultivars was observed with increasing copper ion concentrations in the treatment solution. NIAB 6 seedlings had a lower copper concentration in their root tissues than the Basmati 370 seedlings at all the treatment levels but none of the differences was statistically significant . A higher accumulation of copper in shoot tissues was also observed at higher copper ion concentrations than the control solution (Fig. 4.5). In 16 μM copper ion solution, the amount of copper present in shoot tissue was about 5 times that of the copper present in shoot tissues of seedlings grown in the control solution. A comparison of the cultivars showed that the seedlings of NIAB 6 accumulated lower copper in their shoot tissues than the seedlings of Basmati 370, however the differences were non-significant. In NIAB 6 the accumulation of copper in the shoots of the seedlings grown in the 4 μM copper ion solution was twice ($p < 0.05$) the amount accumulated by the seedlings grown in the control solution, whereas, in Basmati 370, seedlings grown in the 4 μM copper ion solution accumulated 3 times more ($p < 0.05$) copper in their shoot tissues than was accumulated in the shoots of the seedlings grown in the control solution.

4.3. Determination of the lowest copper ion concentration which will induce changes in seedling growth. (Experiment II)

4.3.1. Experimental procedure

The results of the previous experiment showed that low concentrations of copper ions inhibited seedling growth in both cultivars of rice. However, the observed effects were small and often statistically non-significant. It was therefore decided to repeat Experiment I using the same methods as described in Section 4.2.1 but omitting measurements of copper uptake. In experiment II each treatment comprised four specimen tubes instead of eight. The total number of specimen tubes was therefore 96. The experiment was set up in one cabinet only.

4.3.2. Results

The data obtained for the mean length of the longest roots and of the shoots and their fresh and dry weights are presented graphically in Figs. 4.6-4.8. The mean length of the longest roots of the seedlings grown in the treatment solutions followed a similar pattern to that which was observed in Experiment I (Section 4.2.2.).

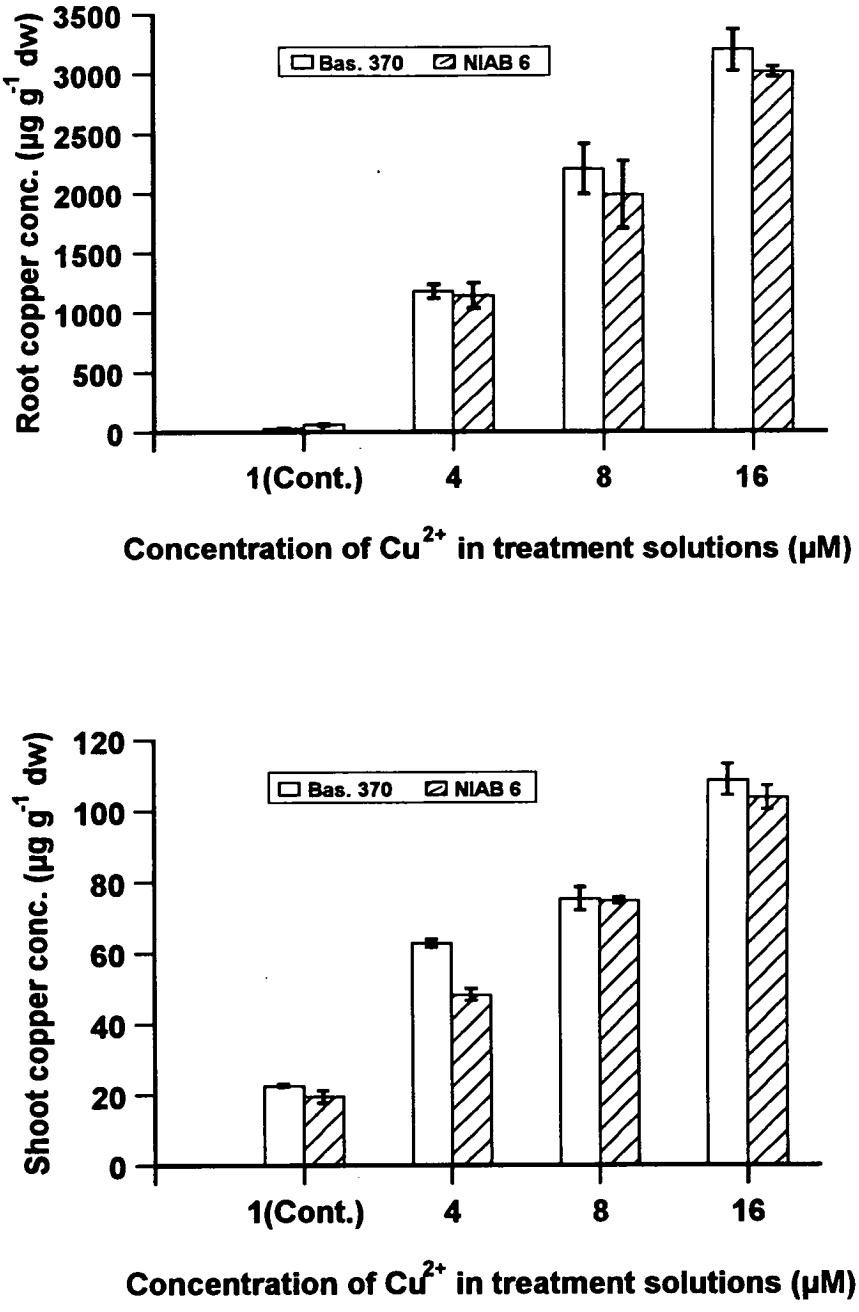


Fig. 4.5. The copper concentration in root and shoot tissue of 20 d seedlings of two cultivars of rice grown at different concentrations of copper ions in nutrient solution. Error bar ± SEM, n=3.

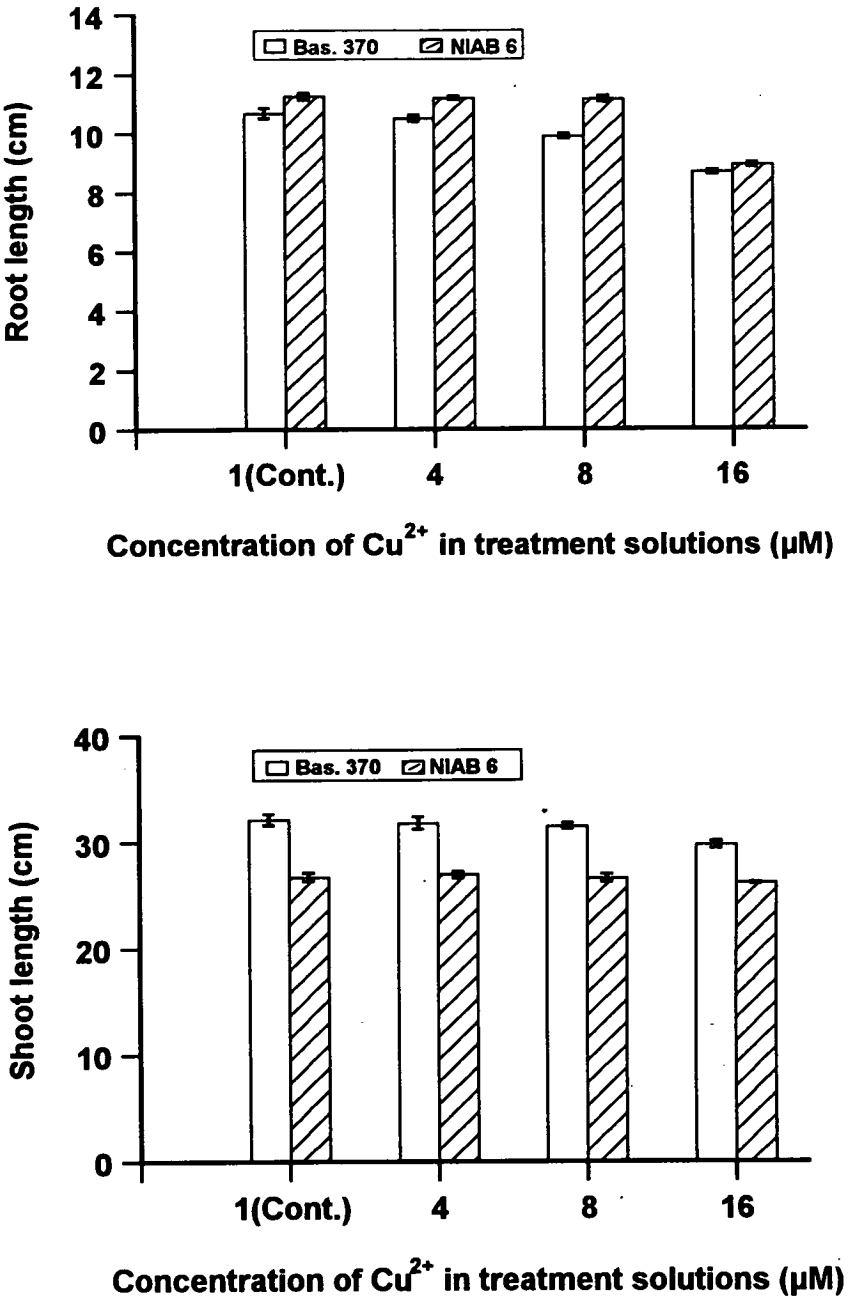
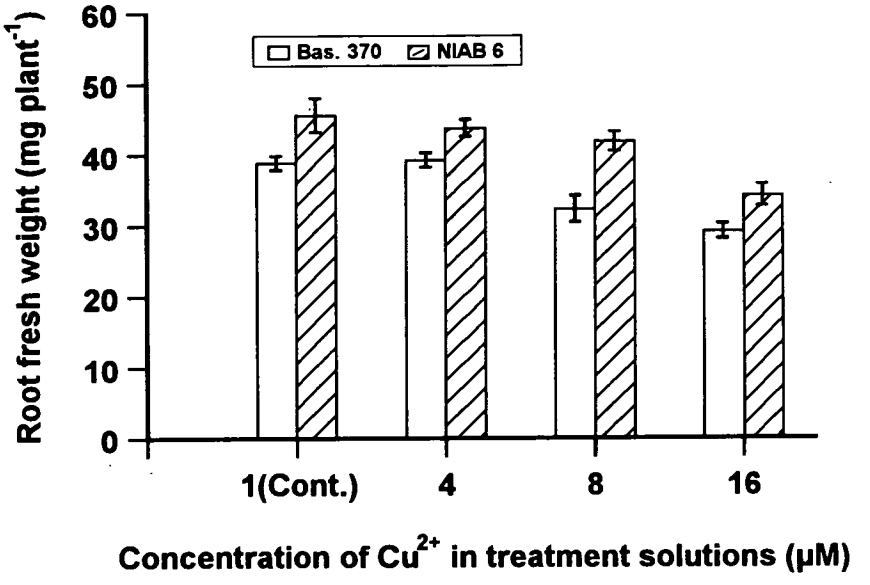


Fig. 4.6. Effect of different concentrations of copper ions on length of the longest root and of the shoot of 20 d seedlings of two cultivars of rice. Error bar ± SEM, n=3. (Experiment II).



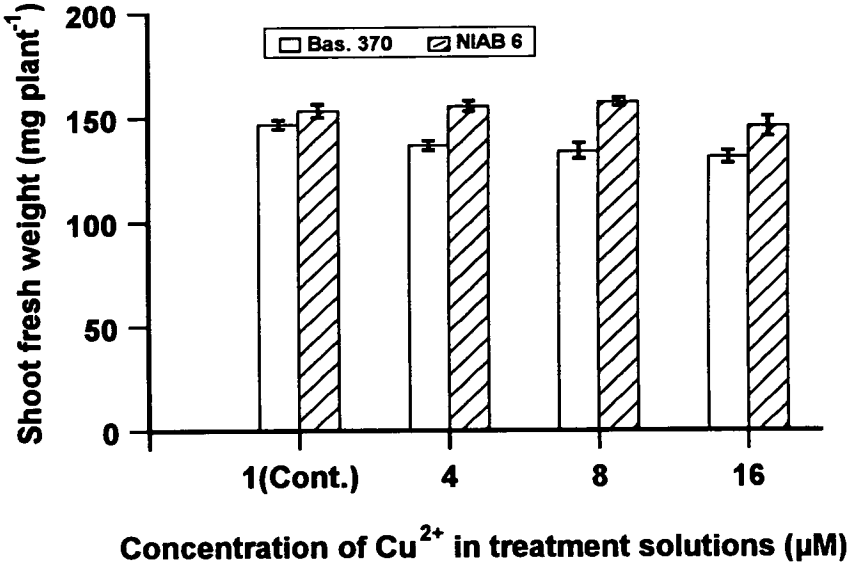


Fig. 4.7. Effect of different concentrations of copper ions on root and shoot fresh weight of 20 d seedlings of two cultivars of rice. Error bar ± SEM, n=3. (Experiment II).

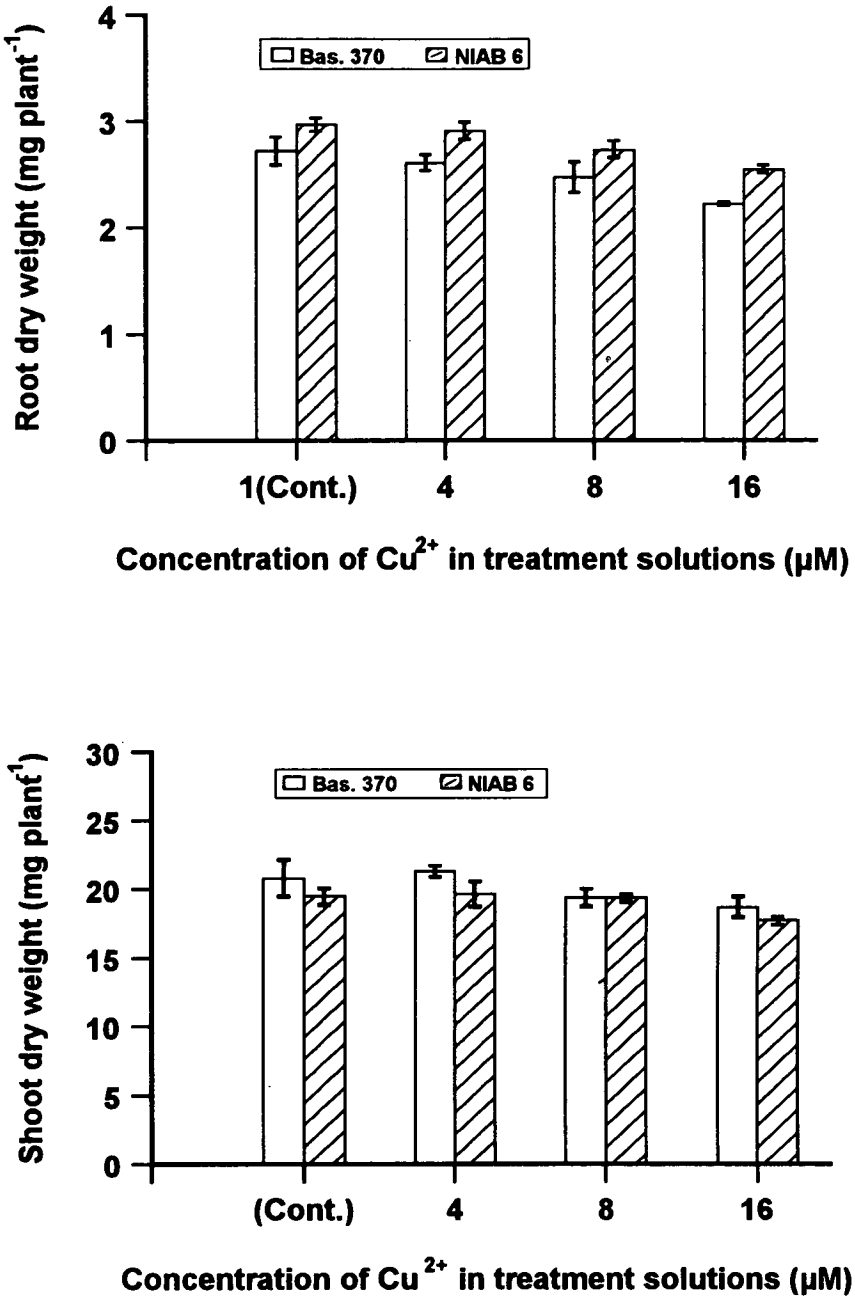


Fig. 4.8. Effect of different concentrations of copper ions on root and shoot dry weight of 20 d seedlings of two cultivars of rice. Error bar ± SEM, n=3. (Experiment II).

However the root lengths in Experiment II were slightly less than the root lengths measured in Experiment I. The effect of the copper ions on the root and shoot fresh and dry weight of the seedlings of the cultivars in Experiment II followed a pattern similar to the results obtained in Experiment I.

4.4. Effect of the pH of the nutrient solution on copper ion-induced changes in seedling growth.

4.4.1. Experimental Procedure

Grains of the rice cultivar NIAB 6 were fixed on to the black polyethylene sheets following the method described in Section 2.2.1. Complete Yoshida nutrient solution of pH 5.5 was prepared as described in Section 2.2.1. Acetate buffers of pH 4.5, 5.0 and 5.5 were prepared using glacial acetic acid and sodium acetate (See Appendix III, for preparation of buffer). The nutrient solution was supplemented with respective buffer solutions to a pH of 4.5, 5.0 and 5.5. The treatment solutions of 1 (control), 8, 16 and 32 μM copper ions were prepared for each solution at the above mentioned pH levels.

An experiment was carried out to determine the effect of acetate on the elongation of the longest root length of the seedlings and to determine the concentration of buffer necessary to maintain the pH of the solution while the seedlings were grown in it. The seedlings were grown at pH 5.5 for 12 d in the following nutrient solutions, control (no buffer), 0.4, 0.6, 0.8, 1.0 and 1.2 mM acetate. The pH of the solutions was measured after 24 h seedling growth, and solutions in the specimen tubes were replaced by the fresh solutions of pH 5.5. After 12 d the root length of the seedlings was measured and the average daily change in the pH of each solution was calculated (Fig. 4.9). On the basis of these results, a concentration of 0.5 mM acetate in the final nutrient solution was selected to get sufficient buffering capacity for 24 h. Buffer in this concentration range did not have any effect on the elongation of the longest root length of the seedlings.

There were two specimen tubes in each treatment and three replications per treatment. Therefore the experiment comprised 72 specimen tubes in total. Two sets of specimen tubes were used for the experiment and the solutions in the specimen tubes were replaced every day. When the seedlings were 15 d old the experiment was harvested. The chlorophyll in the uppermost fully expanded leaf of the seedlings

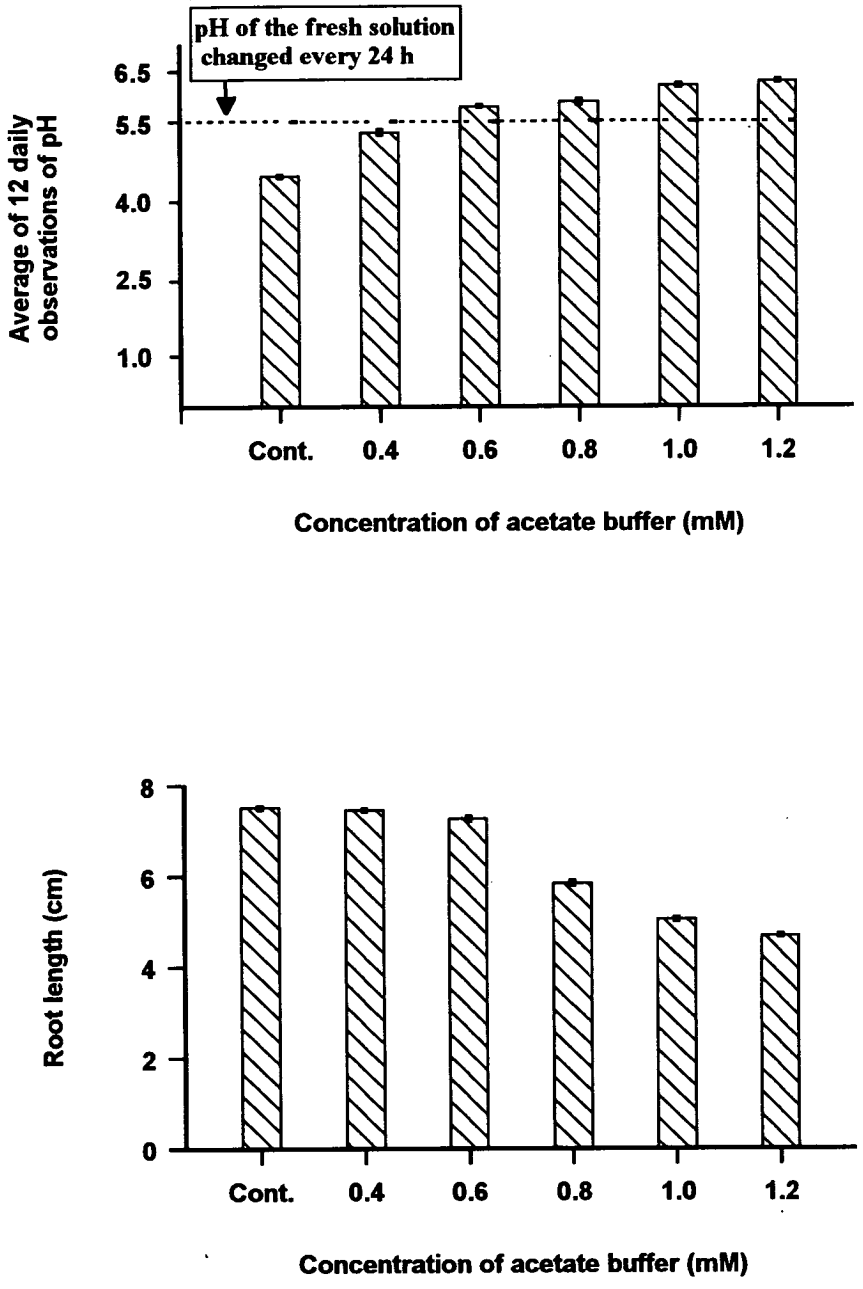


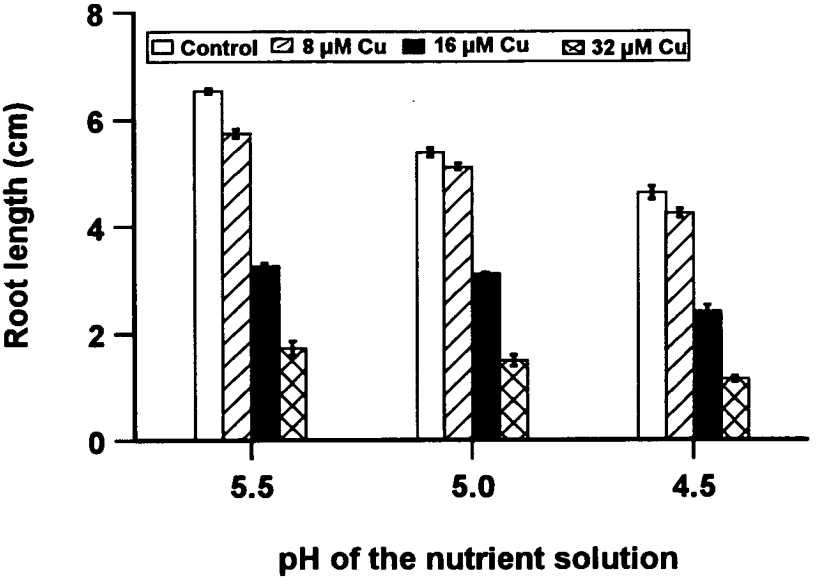
Fig. 4.9. Effect of acetate buffer on pH of the nutrient solution measured 24 h after replacing the nutrient solution with fresh solution at pH 5.5. Root length of the seedlings grown in the solutions supplemented with different concentrations of buffer. Error bar ± SEM, n=3.

was measured by the procedure given in Section 2.5. The length of the shoot and of the longest root was measured, roots were blotted dry and the shoots and roots separated, weighed to obtain fresh weight values, dried and re-weighed.

4.4.2. Results

The figure 4.10 shows the effect of copper ions on the mean length of the longest root and of the shoot of rice seedlings grown in the treatment solutions of different pH levels. The effect of copper ion concentrations on root length i.e. on mean length of the longest root of the seedlings grown in treatment solution at pH 5.5, followed a pattern similar to that which was reported in Section I (Fig. 4.1). The root lengths of the seedlings after growth in the control solutions at pH 5.0 and 4.5 were 18 % and 29 % less ($p < 0.05$) than that of the root lengths of the seedlings grown in the control solutions at pH 5.5, respectively. The effect of pH on the root lengths of the seedlings grown in 8 μM copper ions solutions was similar to that which was observed for the control solutions. The root length of the seedlings after growth in the 16 μM copper ions solution at pH 5.0 was similar to that of the seedlings which had been grown in 16 μM copper ion solution at pH 5.5, but the root length of the seedlings at pH 4.5 in the same concentration of copper ions was 26 % less ($p < 0.05$) than that observed at pH 5.5. Comparison of the copper ion treatments within pH 5.5 showed that the root length of the seedlings grown in the 8 μM copper ion solution was 12 % less ($p < 0.05$) than that of the seedlings grown in the control solution at the same pH. In contrast, the root lengths of the seedlings grown in 8 μM copper ion solution at pH 5.0 and 4.5 were similar to the root lengths of the seedlings grown in the respective control solutions. Interestingly, it was observed that the percentage reduction in the root length of the seedlings, i.e. the difference between the root length in control and 32 μM copper ions treatment, was greater at pH 5.5 than at pH 5.0 and 4.5, respectively. Interaction between copper and pH treatments was non-significant.

Shoot length i.e. the mean length of the shoots of the seedlings grown in the control solution at pH 5.0 and 4.5 was 16 % and 11 % less ($p < 0.05$) than that of the seedlings grown in the control solution at pH 5.5. Similarly the shoot length of the seedlings grown in the 8 μM copper ion solution at pH 5.0 and 4.5 was less ($p < 0.05$) than that of the seedlings grown in the same solution at pH 5.5. The effect of pH on the shoot length of the seedlings grown in 16 μM copper ion solution



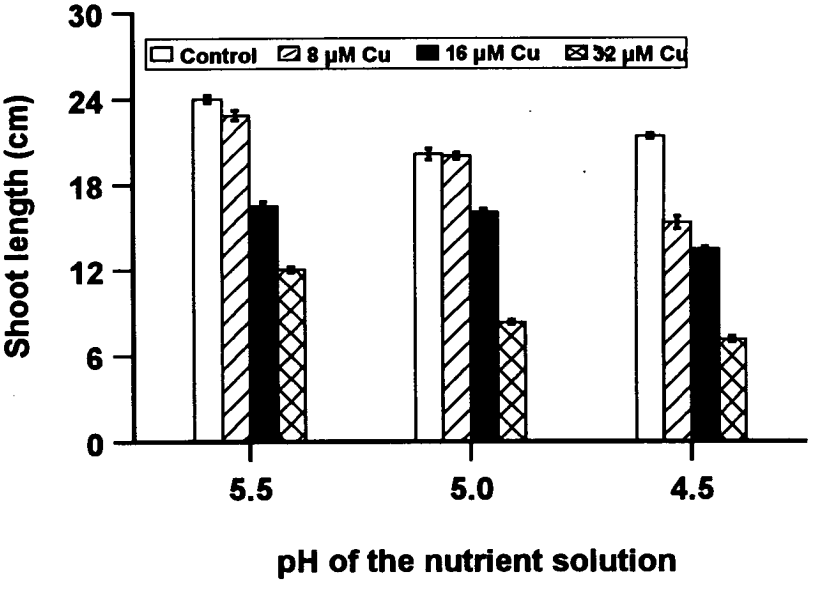


Fig. 4.10. Effect of different concentrations of copper ions and pH of the nutrient solutions on the length of the longest root and of the shoot of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

was significant only at pH 4.5, where the shoot length was 18 % less ($p < 0.05$) than that of the seedlings grown in the same copper treatment at pH 5.5. The comparison of copper ion treatments at each pH showed that the effect of 8 μM copper ion on the shoot length of the seedlings was evident only at pH 4.5 where the shoot length was 28 % less ($p < 0.05$) than that of the control seedlings. The 16 μM copper ion treatment caused a smaller decrease ($p < 0.05$) in the shoot length of the seedlings grown at pH 5.0 compared to the decrease in the shoot length at pH 5.5 and 4.5, when compared to the respective controls. The percentage difference between 32 μM copper ion treatment and the control at pH 4.5 was greater than that between 32 μM copper ion treatment and the control at pH 5.5. This was in contrast to the percentage difference observed in the root length.

The effect of copper ion treatments at different pH levels on the root fresh weight of the seedlings showed a pattern similar to that of the effect on the root length (Fig. 4.11). The root fresh weight of the seedlings grown in the control solutions at pH 5.0 and 4.5 was 27 % and 33 % less ($p < 0.05$) than that of the root fresh weight of the seedlings grown in the control solution at pH 5.5. The effect of the different pHs on the root fresh weight of the seedlings after growth in the 8, 16 and 32 μM copper ion solution was similar to that which was observed for the control solutions. The root fresh weight of the seedlings grown in the 8 μM copper ions solution at pH 5.5 was 13 % less ($p < 0.05$) than that of the seedlings grown in the control solution at the same pH. However, the root fresh weights of the seedlings grown in the 8 μM copper ion solutions at pH 5.0 and 4.5 were similar to the root fresh weights of the seedlings grown in the respective control solutions and this result was similar to the effect of copper ions on the root lengths of the seedlings. The percentage reduction i.e. the percentage difference between the root fresh weight of the seedlings grown in the control and the 32 μM copper ion solutions was greater in the seedlings grown at pH 5.5 than that at pH 5.0 and 4.5.

The shoot fresh weight of the seedlings grown in the control solution at pH 4.5 was 21% and 14 % less ($p < 0.05$) than the shoot fresh weight of the seedlings grown in the control solution at pH 5.5 and pH 5.0, respectively. However the difference between the shoot fresh weight of the seedlings grown in the control solution at pH 5.5 and those grown in the control solution at pH 5.0 was non-significant. The shoot fresh weight of the seedlings grown in the 8 μM copper ion solutions at the three pH values followed a pattern which was observed for control treatment. However, there was no significant effect of pH on the shoot fresh

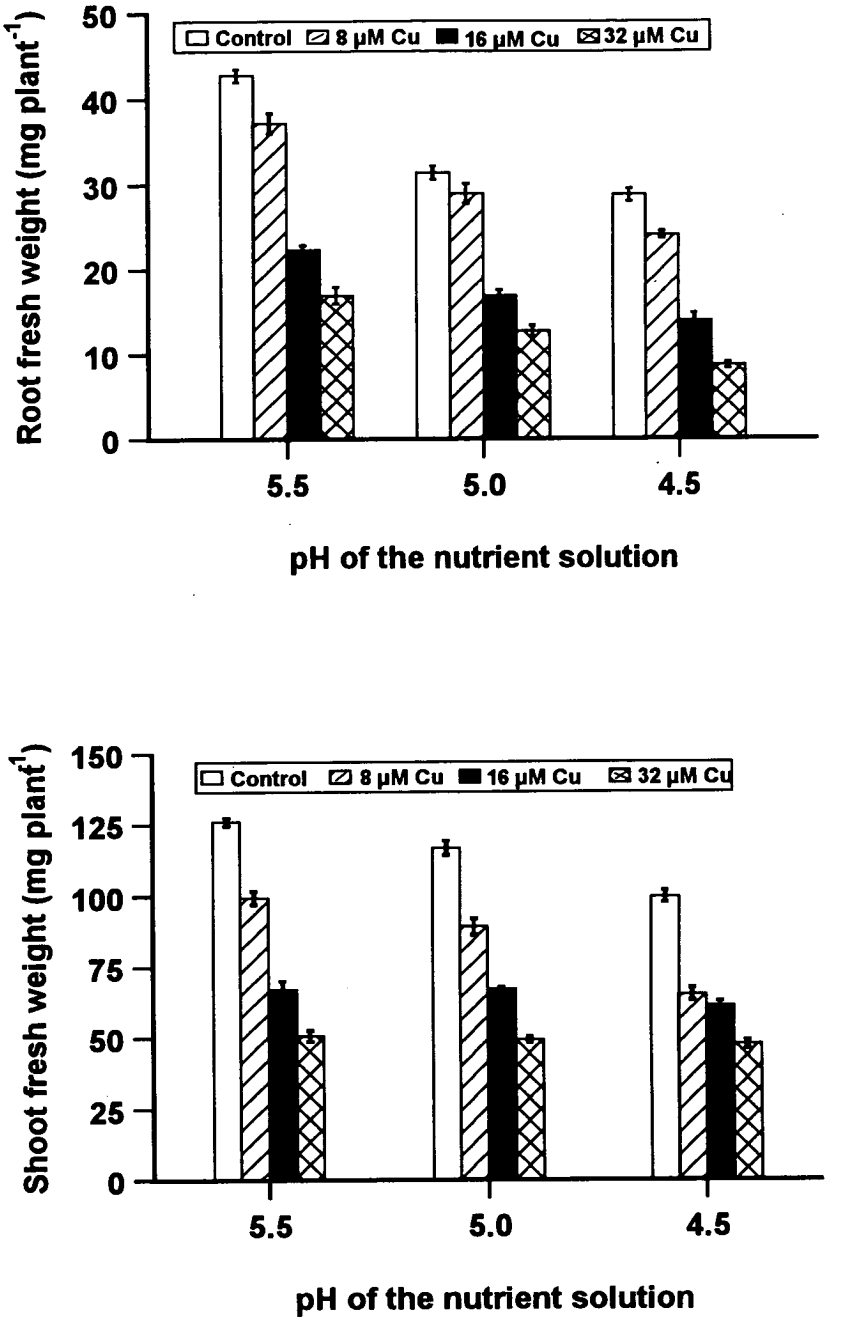


Fig. 4.11. Effect of different concentrations of copper ions and pH of the nutrient solutions on root and shoot fresh weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

weight of the seedlings grown in the 16 and 32 μM copper ion solution. The effect of 8, 16 and 32 μM copper ions on the shoot fresh weight of the seedlings compared to the respective controls, at each of the pH 5.5, 5.0 and 4.5 was significant ($p < 0.05$).

The results presented in Fig. 4.12 show the effect of the copper ion treatments and the pH of the solutions on the root dry weight of the seedlings. The root dry weights of the seedlings grown in the control and the 8 μM copper ions solutions at pH 5.5, 5.0 and 4.5 were similar. The root dry weights of the seedlings grown in 16 μM copper ions solution at pH 5.0 and 4.5 were 27 % and 32 % less than those of the seedlings grown in the respective control solutions.

Shoot dry weight (Fig. 4.12) of the seedlings followed a pattern similar to that which was observed for shoot fresh weight. The shoot dry weights of the seedlings grown in the control solution at 4.5 was 28 % less ($p < 0.05$) than that of the seedlings grown in the same solution at pH 5.5. However the shoot dry weight of the seedlings grown in the 8, 16 and 32 μM copper ions solutions when compared with respect to pH levels showed a non-significant effect of the pH of the solutions. No difference in the shoot dry weight of the seedlings was registered between the 8 and the 16 μM copper ion treatments at any of the pH levels.

Figure 4.13 shows that there was no significant effect of the pH of the solution on the amount of chlorophyll in the upper most fully expanded leaves of the seedlings grown in the control and in the copper ions treatments. However, the amount of the chlorophyll measured from the seedlings grown in the 8 μM copper ions solution at pH 5.5, 5.0 and 4.5 was 45 %, 51 % and 50 % less ($p < 0.05$) than that of the seedlings grown in the respective control solutions. At each pH level the amount of chlorophyll in the uppermost fully expanded leaves of the seedlings grown in the 16 μM copper ions solutions was significantly different from that of the seedlings grown in the 8 μM copper ion solution. The amount of chlorophyll a in the leaves was higher than the amount of chlorophyll b in the leaves of all the seedlings. Copper treatments caused a reduction in the ratio of chlorophyll a/b.

The results of the computer simulation programme GEOCHEM-PC for copper ion speciation at various pH levels of the nutrient solution with added copper ions are presented in Fig. 4.14. It was observed that there was no effect of the

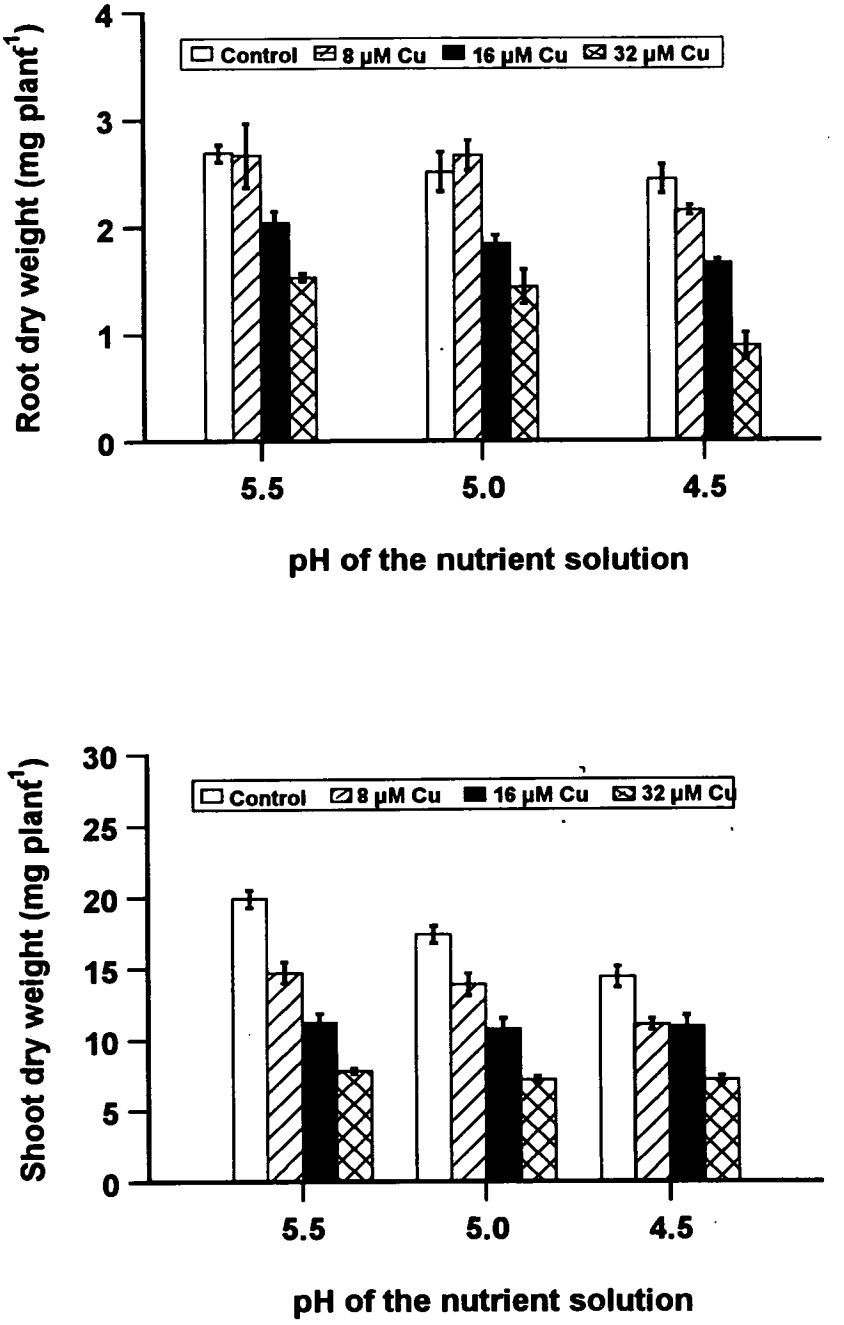
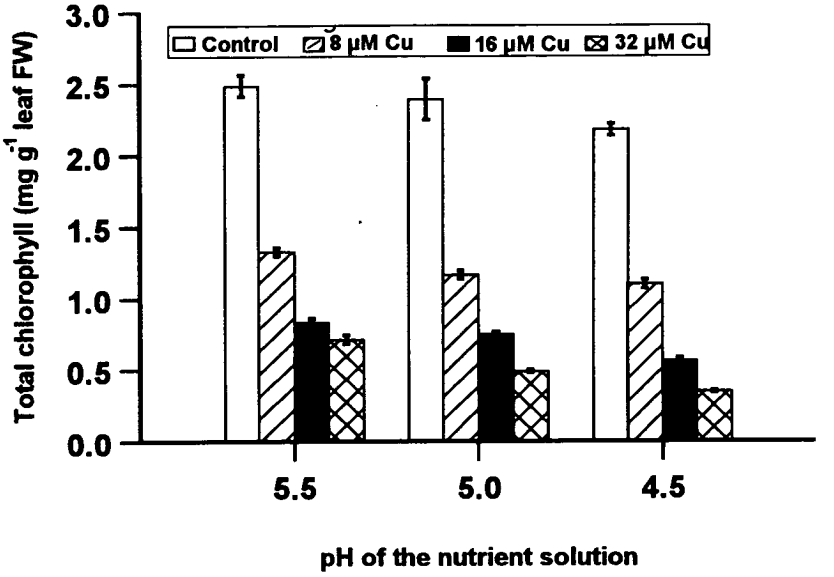


Fig. 4.12. Effect of different concentrations of copper ions and pH of the nutrient solutions on root and shoot dry weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.



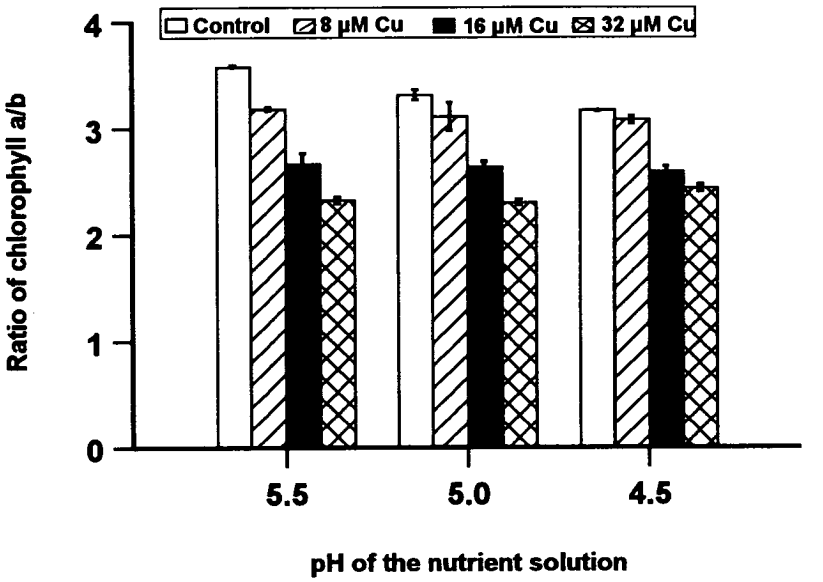


Fig. 4.13. Effect of different concentrations of copper ions and pH of the nutrient solutions on total chlorophyll and ratio of chlorophyll a/b of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

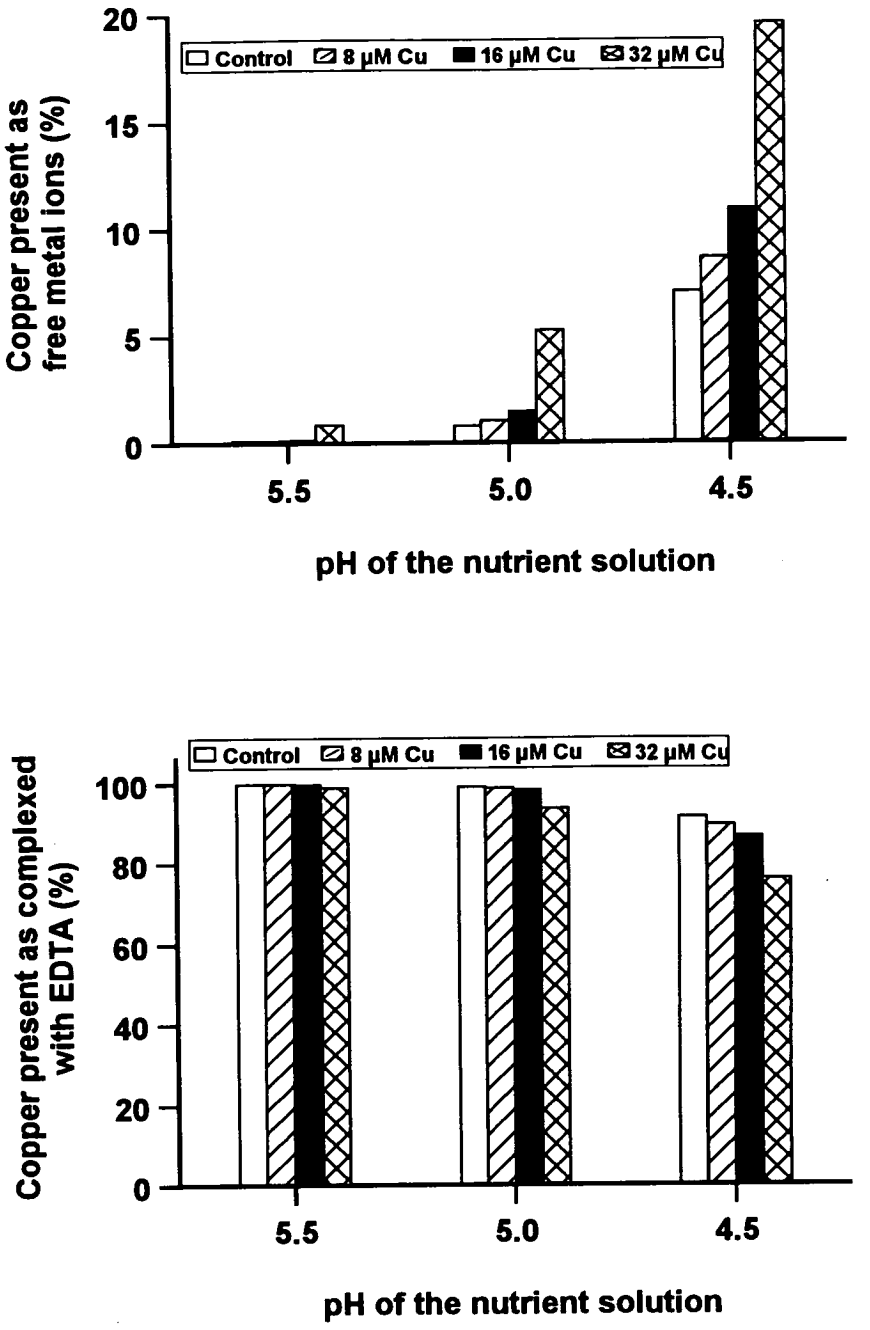


Fig. 4.14. Percentage of added copper ions present in ionic form and complexed with EDTA in complete Yoshida nutrient solution at different pH levels as predicted by GEOCHEM-PC.

addition of acetate buffer on the copper ion speciation in the nutrient solutions at various pH levels compared to the results without the addition of buffer for various pH and copper ion concentrations (data not presented). The control solution contained 1 μM copper, and 7 %, less than 1 %, and less than 0.1 % of that was present in ionic form at pH 4.5, 5.0 and 5.5 respectively. More copper was present in ionic form for all copper treatments at pH 5.0 than at pH 5.5. In 32 μM copper ion treatment at pH 5.0, 5.28 % of the total added copper was present in ionic form. A considerably higher concentration of copper was present in ionic form in solutions at pH 4.5 than the solution at pH 5.5 and 5.0 for all the treatment levels. In 32 μM copper ion treatment about 20 % added copper was present in ionic form when the pH of the solution was 4.5. No precipitation of copper ions was predicted at any of the pH levels of the nutrient solutions and copper ion concentrations. At pH 5.5 about 99 % of the copper was complexed with EDTA even for the highest copper ion treatment i.e. 32 μM copper. When 32 μM copper was added in the solution at pH 5.0 and 4.5, the proportions complexed with EDTA were 80 % and 94 % respectively.

4.5. Measurement of the copper ion-induced leakage of potassium ions, and of root lipid peroxidation.

4.5.1. Experimental Procedure

Grains of both cultivars were stuck to black polyethylene sheets as described in 2.2.1. The rolled sheets were placed in specimen tubes containing full-strength Yoshida nutrient solution and were put in growth cabinets. No copper ions were added to the solutions. There were twelve specimen tubes for each cultivar and the experiment was carried out in three sets.

When the seedlings were 15 d old, they were pre-incubated as described in Section 2.7. After pre-incubation, twelve seedlings with uniform intact root systems were transferred to the specimen tubes containing 50 ml of each of the following treatment solutions prepared in ultra-pure water (2.1.1.): ultra-pure water (control), 4, 8 and 16 μM copper solution prepared from freshly made stock solution (1 mM). Each treatment was replicated three times. The specimen tubes were returned to the cabinet used for seedling growth. The seedlings were exposed for 8 h to treatment solutions for set I, and for 16 and 24 h for sets II and III respectively.

Six seedlings were removed from each treatment and the roots were used for the trypan blue exclusion test as described in Section 2.6.

The remaining seedlings were removed from the solution and roots were separated from shoots. They were blotted dry with paper tissue and root fresh weight was measured. After removal of the seedlings, the volume of the treatment solutions in the specimen tubes was made up to 50 ml by adding ultra-pure water. The absorbance was measured using a potassium filter on a flame photometer (Corning 400). The absorbance of each treatment solution was measured three times and an average was taken. The amount of potassium ions present in the treatment solutions was calculated after comparing the absorbance with standard curve made by KCl standard solutions of known strength. The result thus obtained was converted to μg potassium ions leaked per g root fresh weight.

The seedlings used in the determination of root fresh weights were used for the measurement of TBA-rm (2-thiobarbituric acid-reactive material) accumulation as described in Section 2.8. There were three replications for each treatment. The values thus obtained were converted to absorbance per g root fresh weight.

4.5.2. Results

K^+ ion leakage

In order to determine the effect of copper ion concentration on the leakage of K^+ ions from the whole intact root system of two rice cultivars, analysis of the external solution in which the roots were incubated was done after 8, 16 and 24 h exposure to different treatment solutions of copper ions (Fig. 4.15). The higher the external copper ion concentration in the treatment solution, the greater was the loss of K^+ ions from the roots. A very small amount of K^+ ions leaked into the incubation solution in control (ultra-pure water) treatments in both cultivars during the three incubation periods. After 8 h of incubation of the roots the concentration of K^+ ions detected in the external solution was 7 times greater than that detected in the control solutions. When the roots of the cultivars were exposed to the 16 μM copper ion solution for 8 h, the amount of K^+ ions detected in the external solution was about 91 μg K^+ g^{-1} root fresh weight. The amount of K^+ ions detected in the external solutions after 16 h incubation of the roots of the seedlings with the copper ions solutions was slightly greater than that in the solutions in which the seedlings had been incubated for 8 h and 24 h.

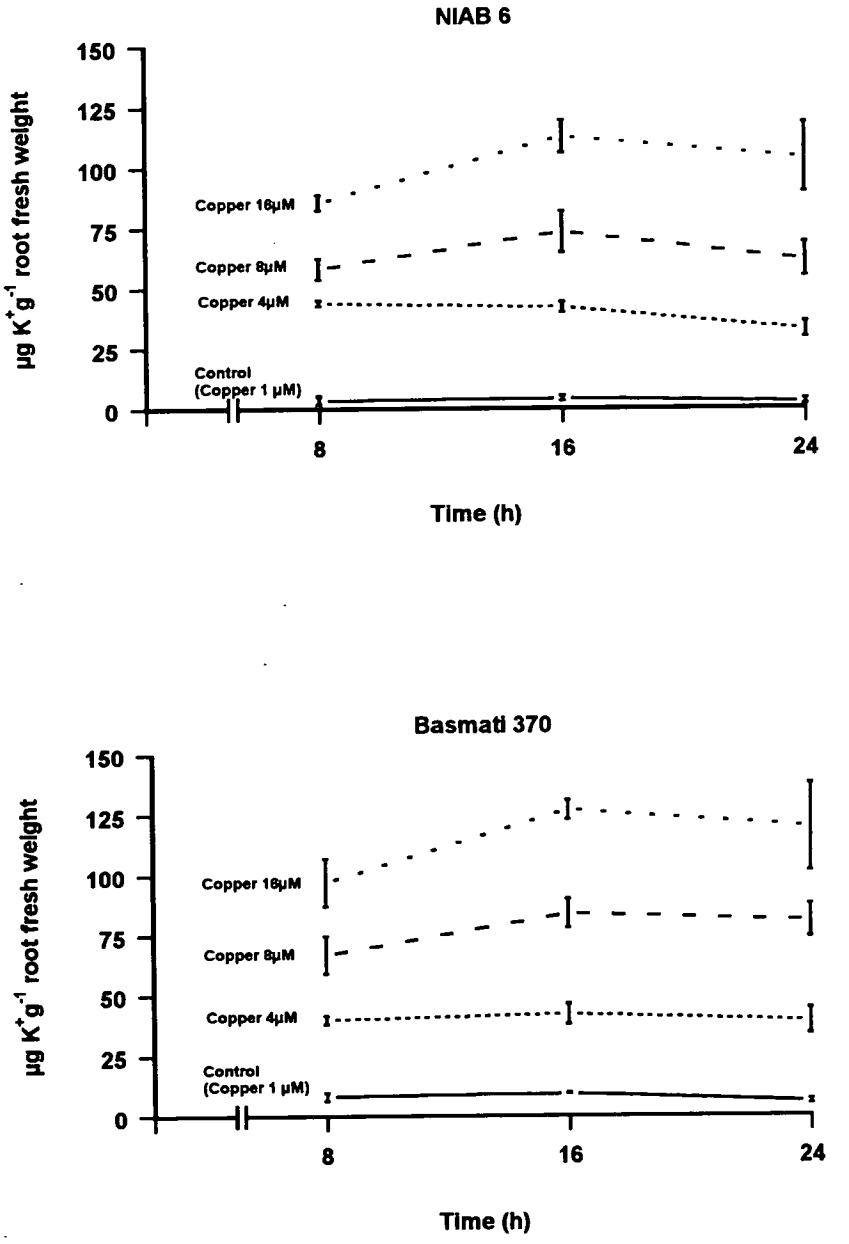


Fig. 4.15. K^+ leakage from the intact root systems of 15 d seedlings of two cultivars of rice determined after three different times of incubation in copper ion solutions. Error bar ± SEM, n=3.

The leakage K^+ of ions from the roots of NIAB 6 was less than the roots of Basmati 370 at all the treatment levels, and over the three specified time periods, except 4 µM copper ion treatment after 8 h of incubation. A maximum value of 140 µg K^+ g^{-1} root fresh weight was detected at 16 µM copper ion solution after 24 h exposure time in Basmati 370. However, the differences between the cultivars were statistically non-significant.

TBA-rm accumulation

The effect of copper ions on lipid peroxidation in the roots was determined by performing an assay for lipid peroxidation products accumulated in roots, based on the level of TBA-rm. The higher the concentration of copper ions in the treatment solutions, the more TBA-rm was found accumulated in the roots (Fig. 4.16). The roots incubated in the control treatment for 24 h accumulated more than double the TBA-rm accumulated by the roots after incubation for 8 h in the same treatment. In all the treatments the increase in the TBA-rm accumulation was almost parallel to the increase in the amount of TBA-rm accumulated in the roots of the control seedlings. The amount of TBA-rm accumulated in the roots incubated for 8, 16 and 24 h in the 16 µM copper ion solutions was about 43 % ($p < 0.05$) more than that accumulated in the roots after incubation for the same periods in the control solution.

When the roots of the seedlings were incubated in different copper ion solutions for 8 and 16 h no difference was registered between the cultivars with respect to TBA-rm accumulation except in Basmati 370 at 16 µM copper after 16 h incubation, where it was higher than that of the 4 µM and 8 µM copper solution. However, after 24 h incubation the TBA-rm accumulated in the roots of the cultivar NIAB 6 was less than that accumulated in the roots of Basmati 370 in the control and in all the treatment solutions.

Trypan blue test

In both cultivars the roots which had been incubated in 16 µM copper treatments showed a deep blue staining in the root cap region irrespective of the length of time of incubation in the copper solution (Plate 4.1). In the 8 µM copper ion treatments there was a slight staining, not typically in the root cap region, but a short distance behind the root tip. This staining was slightly deeper in the roots that had been incubated for 24 h than those which were incubated with copper ion treatment for 8 or 16 h. However, the staining was mainly on the periphery of

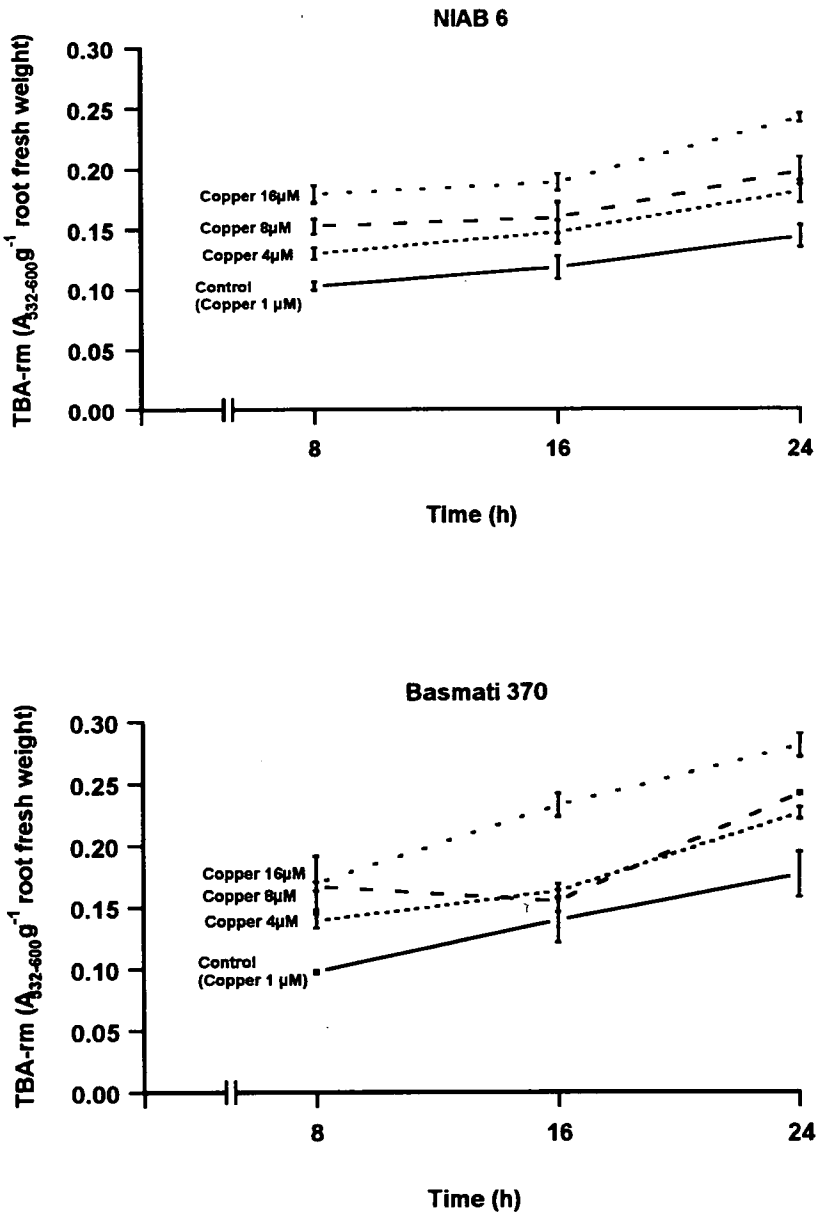


Fig. 4.16. TBA-rm accumulated in the intact root systems of 15 d seedlings of two cultivars of rice after three different times of incubation in copper ion solutions. Error bar ± SEM, n=3.

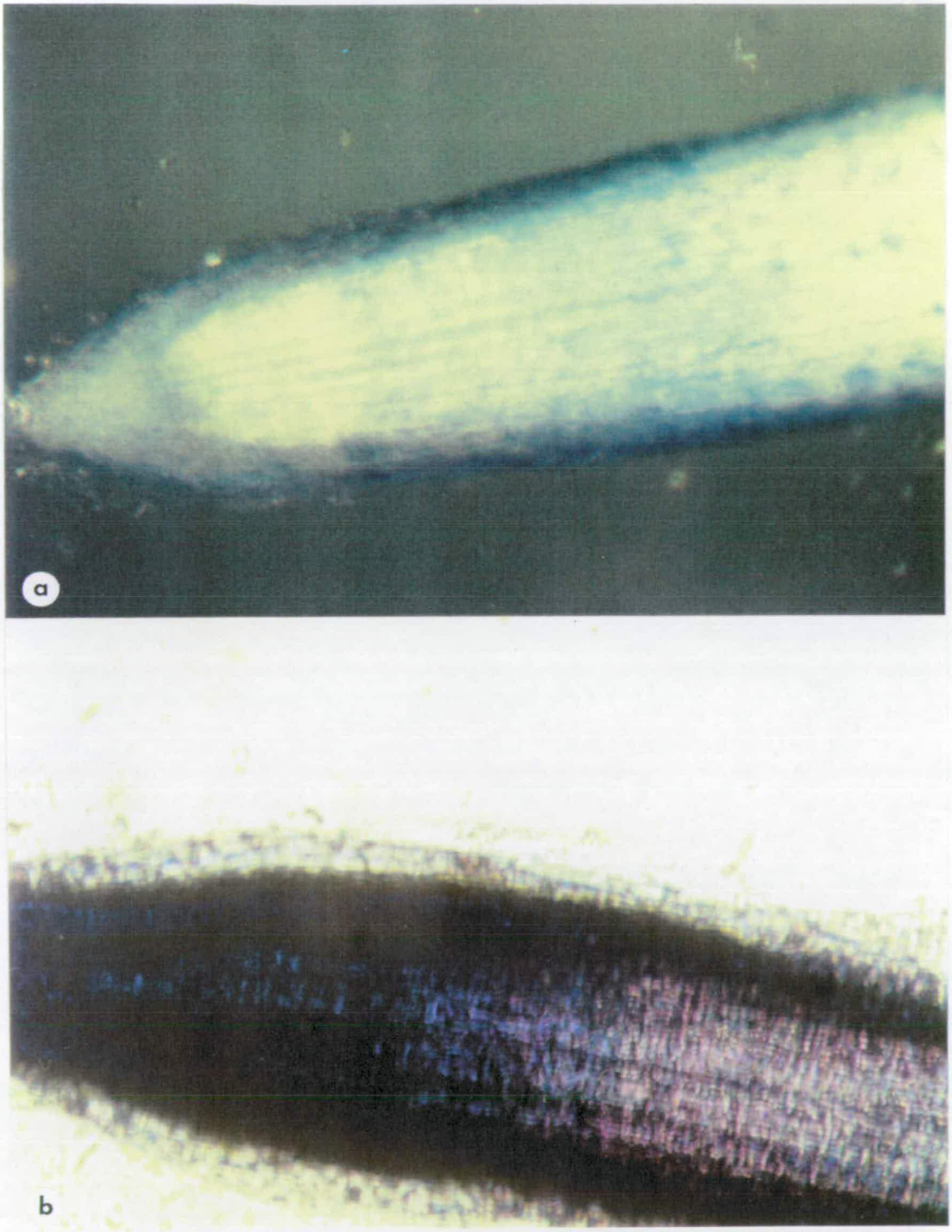


Plate 4.1. Trypan blue staining of the apical regions of roots of 20 d seedlings of rice. (a) Root after 24 h incubation in distilled water. (b) Root after 24 h incubation in 32 μM copper ion solution.

these roots and behind the tip. The roots which were incubated in ultra-pure water (control) and in 4 μM copper showed no accumulation of trypan blue stain.

4.6. Discussion

Effect on growth

The roots of seedlings of cv. NIAB 6 were slightly longer than those of the seedlings of Basmati 370. Significant inhibition in root elongation of both cultivars was observed at 16 μM copper. Similar results were reported by Lidon and Henriques (1992 b), who found that a concentration of 20 μM copper ions in the nutrient solution was toxic enough to inhibit root elongation in seedlings of rice significantly. Their conclusions were based on measurements taken 30 d after the start of the treatment effect. In the present experiment, observations were taken 20 d after the start of the inhibition. NIAB 6 is a short cultivar, whereas Basmati 370 is a tall cultivar therefore an obvious difference in the shoot length of both the cultivars was present. No inhibition in shoot elongation of either of the cultivars was observed in any of the copper ion treatments. Similarly, except for a decrease in root fresh weight of Basmati 370 at 16 μM copper ion, no inhibitory effect of copper on root and shoot fresh weight of either of the cultivars was observed. These observations therefore indicate that root elongation is more sensitive to copper ion toxicity than root and shoot fresh and dry weight. Root and shoot fresh weight of 20 d old seedlings of rice observed in the present investigation closely relates to the root and shoot fresh weight of the 20 d old seedlings of rice grown in different concentrations of copper as reported by Lidon and Henriques (1992 a). The higher susceptibility of root elongation compared to shoot elongation to copper ion toxicity has been confirmed by several authors, when the seedlings were grown both in soil (Baszynski *et al.* 1982) and in solution culture (Stiborová *et al.* 1986; Gupta and Mukherji 1977; Lidon and Henriques 1992 ab).

The results of computer simulation of the composition of treatment solutions showed that more than 99 % of the copper was present complexed with EDTA, which is a readily available form of copper. Both cultivars showed a very similar accumulation of copper in roots and shoots of 20 d old seedlings of rice. However, more copper was accumulated in roots than in shoots. The higher accumulation of copper in the roots is probably due to its reduced translocation to shoots (Fernandis and Henriques 1991). Baker *et al.* (1994) determined the concentrations of twelve metal ions accumulated by 5 populations of *Thlaspi caerulescens* J. & C. Presl, and reported that

there was a wide variation in the extent to which accumulated metal ions are transported from the root systems to the shoot. The amount of copper found accumulated in the roots and shoots of rice seedlings in the present investigation was slightly less than the amount of copper accumulated by the 30 d old seedlings of rice grown in slightly different concentrations of copper ions (Lidon and Henriques 1992 a). The inhibition of root elongation due to copper toxicity, therefore, may be the consequence of high accumulation of copper in the roots. High concentration of copper in the roots may interfere with cell division and/or with cell elongation (Pahlsson 1989).

pH

The effect of copper ion treatments on the growth of rice seedlings in the initial experiments was only observed at the higher concentrations i.e. 8 and 16 μM. Therefore, in order to investigate the influence of the pH of the treatment solution on copper ion toxicity 8, 16 and 32 μM copper ion concentrations were used. The seedlings in the initial experiment were grown for 20 d, while in the investigation of the effect of pH, seedlings were grown for only 15 d because the change in pH of the treatment solution within 24 h due to seedlings growth increased with the age of the seedlings, and it was difficult to maintain the pH of the treatment within a limit of ± 0.2 over the 24 h period. The concentration of acetate which was applied to maintain the pH level of the treatment solutions did not show any toxicity to root elongation of the seedlings. The concentration of acetic acid in soil can exceed 10 mM, which is considered to be toxic to the roots of many plants and this problem becomes more serious on soils with pH values below about 5.5, as undissociated acids are more toxic than their anions (Wild 1988).

Significant inhibition in root elongation at 8 μM copper ions and pH 5.5 was observed, whereas, in the previous experiments (experiments I & II) the same treatment showed a non-significant effect on root elongation. This could be due to the effect of acetate buffer causing increased toxicity of copper ions. Lynch (1980) observed no stimulation of root elongation in roots of barley at 1 mM acetic acid when the pH of the solution was 5.5, however, Gudjónsdóttir (1966) has reported that 1 mM acetic acid promoted the growth of excised roots of wheat seedlings at pH 5.5 while the same concentration inhibited root elongation at pH 5.0. Gussin and Lynch (1982) reported a stimulation of elongation of root tips of barley caused by treatment with 5 mM concentrations of acetic acid. However, treatment of whole roots with the same concentration of acetic acid showed no effect and at higher

concentrations, root growth was inhibited. An inhibition in the root elongation of the seedlings grown in control solution at pH 5.0 and 4.5 was also observed compared to that of the control seedlings at pH 5.5. This shows that lower pH was itself toxic to the root elongation of rice seedlings. Furthermore, root length was less at pH 5.0 and 5.5 but inhibition was more at pH 5.5. Dijkshoorn *et al.* (1981) and Sanders *et al.* (1986) found copper more toxic to the growth of ryegrass at pH 4.5 than at pH 7.0. In contrast, Tanaka *et al.* (1982) and Nasu *et al.* (1983) reported that growth of *Lemna paucicostata* plants was more strongly inhibited due to copper toxicity at pH 5.1 than at pH 4.1.

The amount of chlorophyll in the uppermost fully expanded leaves was less in seedlings grown in solutions of copper ions than in the control seedlings. About 50 % less chlorophyll was found in seedlings which had 8 μM copper ion treatments than in control seedlings. These results confirmed the findings of Lidon and Henriques (1991) who reported a similar decrease in the amount of chlorophyll in the leaves of 30 d old rice seedlings grown in toxic concentrations of copper ions. In the present investigation it was observed that, in contrast to the effects of pH on the root elongation, there was no effect of the pH of the treatment solution on the amount of chlorophyll in the leaves. Furthermore, the effect of copper ion toxicity on the amount of chlorophyll was not affected by change in the pH of the treatment solutions. Tanaka *et al.* (1982) reported that there was no difference in the concentration of chlorophyll in the *Lemna paucicostata* plants grown on a copper-free medium at pH 4.1 than in those grown on the same medium at pH 5.1. However, on addition of copper to the growth media, less chlorophyll was found in those plants which were grown at pH 5.1 than in those which were grown at pH 4.1.

The pH of the nutrient solution may exert its effect on plant growth either directly by affecting uptake sites in roots or indirectly by changing the chemical speciation of the dissolved metal pool (Peterson *et al.* 1984). The free metal ions are generally considered to be the major toxic species (Anderson and Morel 1970). In the present study, the computer simulation programme GEOCHEM-PC showed that the proportion of copper ions in the ionic form was greater in all the solutions at the lower pH levels than at at pH 5.5. However, the amount of copper in the ionic form was only a small fraction of the total copper. Peterson *et al.* (1984) have also reported an increase in the free copper ions at low pH. These authors attributed the influence of pH on copper toxicity to the competition between H^+ and Cu^{2+} for cellular binding sites, and concluded that higher copper ion toxicity occurs at higher

pH where competition with H^+ is low. On the contrary, Moore and Ramamoorthy (1984) proposed that, since a lowering of pH increases the proportion of free ions in solution, toxicity therefore, would be greater at low pH. When inhibition in root elongation of the seedling due to copper ion toxicity relative to the control was calculated, it was observed that more relative root growth inhibition occurred at pH 5.5 than at pH 5.0 and 4.5. Therefore it was concluded that further investigations on the effects of copper ions on seedling growth should be carried out at pH 5.5.

K^+ leakage and lipid peroxidation

The trypan blue staining test is routinely used as a measure of cell viability (Moldéus *et al.* 1978). When the tissue is immersed in the solution of trypan blue, staining occurs only in those cells in which the plasmamembrane of the cells of the roots has been damaged. The roots of rice seedlings after incubation with 16 µM copper ions showed a deep staining in the root cap and apical meristem. Exclusion of trypan blue from the roots of seedlings incubated in ultra-pure water and 4 µM copper ions showed that the plasmamembranes of the root cells were intact. In the roots of the seedlings which were incubated with 8 µM copper, staining was absent from the central root cap cells and was observed only in the outer cell layers of the root. When the roots were incubated in 8 µM copper ion solution for 24 h, the staining was deeper than when they were incubated in the same copper ion solution for 8 h. This could be due either to an increase in copper-induced damage or to greater uptake of the stain during the longer period or both. De Vos *et al.* (1989) incubated roots of copper-tolerant *Silene cucubalus* seedlings with 100 µM copper ions for 24 h and then stained with trypan blue to see the effect of copper ions on the plasmamembrane integrity. Between 3 and 6 h of incubation, they observed staining in a zone at 5-10 mm distance away from the tip-zone, whereas after 24 h of incubation roots were heavily stained over a length of about 10 mm, including the apical zone. Both the cultivars used in the present investigation showed no differences in the staining pattern. De Vos *et al.* (1991) demonstrated that after incubation of roots with copper ions a copper-tolerant and a copper-sensitive cultivar of *Silene cucubalus* could be differentiated on the basis of trypan blue staining.

Incubation of roots of rice seedlings with copper ions caused leakage into the external solution. A small amount of potassium was lost from the roots of control seedlings, incubated in ultra-pure water. A similar response has been observed by Wainwright and Woolhouse (1977) who observed some potassium leakage in the control treatment of both copper-sensitive and copper-tolerant clones of *Agrostis*

tenuis. The leakage of potassium ions from roots incubated in ultra-pure water could be a stress response. The amount of potassium ions leaked as a result of such stress remained unchanged over the incubation period, suggesting that this small leakage is a general stress response. The higher the concentration of copper ions in the treatment solution the higher was the amount of potassium ions released into the external solution. However the amount of potassium ions recorded after 24 h of incubation, for each treatment of copper ions, was almost the same as that recorded after an 8 h incubation period. Therefore, it appears that the effect of copper ions on roots was dose-dependent rather than time-dependent. De Vos *et al.* (1991) reported a similar finding where the initial loss of potassiun ions due to copper ion toxicity was higher at higher copper ion concentrations. The loss of potassium ions due to copper ion treatments cannot be attributed simply to extracellular Cu^{2+}/K^{+}-exchange, because extracellular potassium had been replaced with calcium or sodium during pre-incubation. Furthermore, the amount of potassium ions measured in the external solution is too high compared to the copper ions applied to be accounted for by a simple cation exchange mechanism (De Vos *et al.* 1989). Therefore it appears that upon incubation of roots of rice seedlings with copper ions, damage to the plasmamembrane of root cells occurred which caused leakage. This conclusion confirms the findings of Wainwright and Woolhouse (1977) working with *Agrostis tenuis*, De Vos *et al.* (1989) working with *Silene cucubalus* and Strange and Macnair (1991) working with *Mimulus guttatus*. In the present investigation, there was no difference between the cultivars in the pattern and the amount of potassium ions leaked. Wainwright and Woolhouse (1977) reported a higher amount of potassium ion leakage from the roots of copper-sensitive than copper-tolerant clones of *Agrostis tenuis*, incubated with a range of concentrations of copper ions. Similarly in *Silene cucubalus*, copper ion-induced leakage of potassium ions was greater in a copper-sensitive cultivar than in a copper-tolerant cultivar (De Vos *et al.* 1991). Potassium is vital for turgor maintenance. Therefore, any loss of potassium ions might result in decreased turgor in roots and thereby affect root elongation.

The permeability of membranes can be affected by lipid peroxidation (Dhindsa *et al.* 1981). The amount of lipid peroxidation products accumulated in the roots of rice seedlings during a period of treatment would therefore give an indication of the effects of the copper ion treatments on the permeability of the membranes of cells of roots. Accumulation of lipid peroxidation products was observed even in the roots of seedlings which were incubated in ultra-pure water. An increase in the accumulation of lipid peroxidation products during incubation in isolated microsomes

in the absence of copper ions (De Vos *et al.* 1989) and in intact root systems of *Silene cucubalus* seedlings in control treatments has also been reported (De Vos *et al.* 1991). However, the amount was very much higher in seedlings incubated in the presence of copper ion. There was a steady increase in the accumulation of these products over the 24 h treatment period. This effect may be attributed to the continuation of lipid peroxidation in the internal membranes of the cells. Copper-induced peroxidation in membrane lipids is associated with extensive degradation of intracellular membranes and organelles (Mattoo *et al.* 1983). Lipid peroxidation in isolated chloroplasts from spinach leaves (Sandmann and Böger 1980) and isolated microsomes from roots of *Silene cucubalus* (De Vos *et al.* 1989) due to copper ion toxicity has been demonstrated. Potassium ion leakage from intact excised root systems of *Agrostis tenuis* was observed within 1 h (Wainwright and Woolhouse 1977) and in roots of *Silene cucubalus* as early as within 15 min (De Vos *et al.* 1989) of exposure to copper ions. This means that lipid peroxidation of the plasmamembranes of the cells of roots resulting in its leakiness starts immediately after the roots are exposed to toxic concentrations of copper ions and afterwards it continues in the internal membranes. To cause leakage of potassium ions very slight damage to the plasmamembrane of the cells of the roots would be enough. This effect might be responsible for much of the potassium ion leakage measured after 8 h of treatment application in the roots of rice seedlings than the amount of potassium ions leaked over the 24 h period. Subsequent lipid peroxidation in the membranes of the intracellular organelles, which do not contribute much towards the loss of potassium ions might be responsible for increased TBA-rm accumulation. This continuation of lipid peroxidation in the internal membranes may ultimately result in complete disintegration of root structure and function.

Peroxidases are thought to be responsible for lipid peroxidation. Their induction is considered as a general response of higher plants to various stresses, such as, wounding, flooding, drought, metal ion toxicity etc. (Pandolfini *et al.* 1992). An increase in peroxidase activity has been observed in the roots and leaves of various plant species after the application of toxic doses of copper ions (Van Assche and Clijsters 1990). In addition to the effect on membrane lipids, peroxidases may also be responsible for the degradation of indole acetic acid which is thought to be directly involved in the process of root elongation (Coombes *et al.* 1976).

5. COPPER ION-INDUCED MODIFICATIONS IN THE MORPHOLOGY OF THE ROOTS OF RICE *(Oryza sativa* L.*)* SEEDLINGS.

5.1. Introduction

Almost all higher plants have roots, though the root:shoot ratio varies considerably. For example, Spanish moss, *Tillandsia usneoides,* is very nearly rootless in the adult state, whereas members of the Podostemaceae and some orchids (e.g. *Taeniophullum*) are nearly all root when in the vegetative phase (Barlow 1990). Almost all roots are cylindrical. The apical meristem of the root is protected by a thimble-like root cap which covers the root tip. Growth in length takes place near the root tip. Behind the root tip there is a zone of cell division, behind which there is a zone of cell elongation and then a zone of cell differentiation. There are no sharp boundaries between these zones. Most cell growth takes place in the zone of cell elongation. Here the cells elongate to more than ten times their original length. Cells become functionally mature in the zone of cell differentiation, which is sometimes known as the root hair zone.

Root hairs, which arise as papillae on epidermal cells are tubular extensions of the root epidermal cells and occur as a result of lateral growth of the cell. Root hairs vary in length from 80 to 1500 μm, and in diameter, from 5 to 20 μm. The shortest hairs are near the distal end of the hair zone (towards the apex), whereas the longest are near its proximal end (towards the base) (Jaunin and Hofer 1986). Root hair density varies from 50 to 500 million per m^2 of root surface (Dittmer 1949). Usually, root hairs are short-lived cells, collapsing and being worn out after a few days or weeks (Cutter 1970). Root hair growth can increase the area of the outer surface of the epidermal cells by 2 to 10 times. There is a wide variation between species in the number and length of root hairs, most species have root hairs, a few have none or extremely small root hairs. Root hairs are involved in the uptake of nutrients (Nye 1966; Itoh and Barber 1983; Clarkson 1991) and their uptake efficiency is correlated with their length (Itoh and Barber 1983). In roots growing in control nutrient solutions, root hairs are dense and their length increases regularly from tip to the proximal parts of the root. Inhibition of the formation of root hairs in *Betula* seedlings was reported by Patterson and Olson (1983) when seedlings were grown in solution containing toxic concentrations of copper ions. Lidon and Henriques (1992 a) while studying the effect of copper ion concentrations on the growth of rice seedlings, observed that when the seedlings were grown in nutrient

medium containing 100 μM copper ions, no root hairs were present on the seminal roots and the formation of secondary roots was also inhibited. Similarly, Brady *et al.* (1993) observed that when soybean seedlings were grown in nutrient solution containing 2 μM aluminium, root hairs failed to develop or were slow in emergence. Kumar *et al.* (1993) reported not only a decrease in the size of the root hairs of *Sesamum indicum* when seedlings were grown in a solution of lead ions but also a decrease in the number of the root hairs as well. The hair zone of most roots is 1 to 4 cm long and is situated just behind the zone of active cell elongation (Jaunin 1988). Brady *et al.* (1993) reported a decrease in the length of the root hair-zone in seedlings grown in 2μM aluminium solution compared to the length of the root hairzone of the seedlings grown in a control solution.

When viewed as a cross-section, most roots can be subdivided into epidermis, cortex, endodermis, and stele (vascular cylinder). The innermost layer of the cortex is the endodermis, a cylinder, one cell thick that forms the boundary between the cortex and the stele. The number of rows of cortical cells between the epidermis and endodermis varies with plant species but is usually in the range of 5 to 10. Branch roots arise in the pericycle at the periphery of the vascular cylinder and push through the endodermis, cortex and epidermis. Later, the xylem, phloem, endodermis and cortex of the branch root become continuous with those of the main root.

The cells present at the periphery of the root cap produce mucilage. In addition to the production of mucilage, these cells are continually sloughed off, so that the surface of the growing root becomes covered by very heterogeneous material. The slime produced by various hydroponically-grown plants contains large quantities of galactose, arabinose, xylose, galacturonic acids and fucose (Rougier 1981; Chaboud 1983; Chaboud and Rougier 1984). These compounds possess such properties as adhesiveness, water holding capacity and the ability to associate with other molecules. Therefore, production of mucilage by root cap cells and, to some extent also by epidermal cells, may have an important adaptive role (Barlow 1990). In hydroponic experiments on cowpea, removal of the root cap mucilage resulted in an enhanced sensitivity of root elongation to aluminium (Horst *et al.* 1982). These workers also reported a greater accumulation of aluminium by the root apex and mucilage than by other parts of the root. Ryan *et al.* (1993) investigated the spatial sensitivity of maize roots to aluminium toxicity by a divided-chamber technique. They reported that the root cap had no effect on the aluminium-induced inhibition of

root elongation in solution experiments, and found that the root meristem was the primary site of the toxic effect and a shorter zone of cell division was present compared to the control. Meristem length i.e. the distance from the root cap boundary to the proximal boundary of the meristem, in seedlings of *Festuca rubra* was much shorter after 12 h of exposure to 80 μM zinc and continued to decline over the subsequent 84 h compared to the meristem length of the seedlings grown in control solutions (Powell *et al.* 1986; Davies *et al.* 1991). This smaller root meristem indicates that metal ion toxicity changes the balance which normally exists between cell production and the onset of cell elongation in the root apex (root cap, meristem and elongation zone). An inhibition in root length due to any stress factor generally results in a shorter meristem zone (Rost and Baum 1988). There is no published information about the similar effects of copper ions on these aspects of root development in rice.

Davies *et al.* (1991) investigated the effect of zinc ions on the number of root primordia in the roots of *Festuca rubra* seedlings. They found that incubation of roots in 80 μM zinc solution resulted in the formation of more root primordia than in the control seedlings. The roots in the control seedlings were longer than those in the zinc treated seedlings and so the primordia were formed much closer to each other in the zinc treated roots than in the control roots. As the meristem zone was shorter in treated roots, differentiation of lateral root primordia occurred nearer the apex, causing branching near the root tip. These workers considered that although there was an increase in the number of lateral root primordia in the roots treated with 80 μM zinc, lateral roots would be unlikely to perform normally, as they would be growing in the same toxic environment as the primary root.

Copper ion-induced changes in the growth of rice seedlings have been described by many workers. For example, Mukherji and Gupta (1972) studied the effects of copper ions on root and shoot growth and Lidon and Henriques (1992 ab) have reported the effects of copper ions on seedling growth and biomass yield. Roots have been observed to be more sensitive to copper ion toxicity than shoots. This effect has also been observed in Section 4, where root growth of rice seedlings was significantly inhibited at lower copper ion concentrations than shoot growth. The effects are seen initially, as abnormal growth and the inhibition of root elongation. Inhibition of the length of the longest root of seedlings grown in media containing toxic levels of copper ions is the one of the most sensitive parameters and has been used to give an indication of the degree of metal ion toxicity (Wilkins 1957, 1978).

Copper uptake, translocation, accumulation in the cells, effects on cell biochemistry and on plant physiology and morphology have been thoroughly reviewed by Fernandes and Henriques (1991). Recently, subcellular localisation of copper ions has been studied by Lidon and Henriques (1994) in roots of 30 d old rice seedlings grown in nutrient solution containing different copper ion concentrations. They reported the presence of large amounts of electron-dense copper-containing deposits inside the root cell vacuoles. Comparatively little is known about the morphological changes in roots that accompany these effects. There are no quantitative studies about the copper ion-induced changes in root morphology in rice seedlings. In the present study, root morphology was investigated by light and scanning electron microscopy of roots grown in control solutions and in 20 μM copper ion solution.

5.2. Experimental Procedure

Grains of rice cv. NIAB 6 were fixed in the slits in black polyethylene sheets as described in Section 2.2.1. Complete Yoshida nutrient solution at pH 5.5 served as the control (1μM copper). The other treatment was 20 μM copper ion present in complete Yoshida nutrient solution at pH 5.5. The sheets containing seeds were placed inside the specimen tubes and put in the cabinets under the growth conditions described in Section 2.2.1. There were six specimen tubes for each treatment and three replications per treatment. Two sets of specimen tubes were used and the solutions in the specimen tubes were replaced every day. Observations for various parameters were made 4, 8 and 12 d after the start of the experiment. The number of roots and the lengths of the longest root were measured on the same seedlings at three time intervals.

The longest root of each seedling was cut off at the point of attachment to the shoot, placed on a glass slide in a few drops of distilled water and covered by a glass cover slip. The length of the root hairs at a distance of 95 and 180 μm from the root apex was measured under the light microscope using an eye-piece graticule calibrated using a standard micrometer slide. All measurements were made at a magnification of x 100. The distance from the root apex to the start of the hairy-zone was measured. The distance from the root apex to the point of emergence of the youngest lateral root on the longest root was also measured by the same procedure. Root samples were taken for cryopreservation, cryosectioning and replica preparation as described in Sections, 2.9.1-2.9.3. Electron micrographs of each treated root were taken along the root axis from apex towards base using a

Cambridge Stereoscan S250 electron microscope. In each treatment, three specimens were photographed. A template of 2.5 cm x 5 cm was placed in the centre of the electron micrographs of specimens between 0.5-0.6 mm distance from the root apex to measure the length, width and the number of cells present in that template, and measurements were made using a Hisketch 1212 digitising pad.

5.3. Results

Root length i.e. mean length of the longest roots of 4, 8 and 12 d old seedlings of NIAB 6 grown in the control and the 20 μM copper ion solutions is presented in Fig. 5.1. There was a linear increase in the root length of the seedlings grown in the control solution. The root length of the seedlings grown in the 20 μM copper ions solution for 4 d was 20 % less than that of the seedlings grown in the control solution for 4 d. The root length of the seedlings after 8 d and 12 d growth in the 20 μM copper ions solution was 45 % and 43 % less ($p < 0.05$) than the root length of the seedlings grown in the control solution for the same number of days.

The data presented in Fig. 5.1 show that the number of roots increased with time and the seedlings grown in the 20 μM copper ions solution had fewer roots per plant than the seedlings grown in the control solution. The effect of copper ions was non-significant after 4 and 8 d and the number of roots in the seedlings grown in the 20 μM copper ions solution after 12 d was 43 % less ($p < 0.05$) than the number of roots of the seedlings grown in the control solution.

Measurements of the length of the root hairs were taken at a distance of 95 μm from the root apex of the longest root of the seedlings after 4, 8 and 12 d growth in the control and 20 μM copper ion solution (Fig. 5.2). The seedlings grown for 8 d and 12 d in the control solution had shorter root hairs than seedlings grown for 4 d in the same solution. The lengths of the root hairs in the seedlings grown in the 20 μM copper ions solution for 4 and 8 d were 70 % and 88 % less, respectively ($p < 0.01$) than those of the seedlings grown in the control solution. The length of the root hairs after 8 d seedling growth in the 20 μM copper ion solution was 72 % less ($p < 0.01$) than that of the root hair length measured when the seedlings were 4 d old. No root hairs were present at a distance of 95 μm from the apex after 12 d seedling growth in the 20 μM copper ion solution.

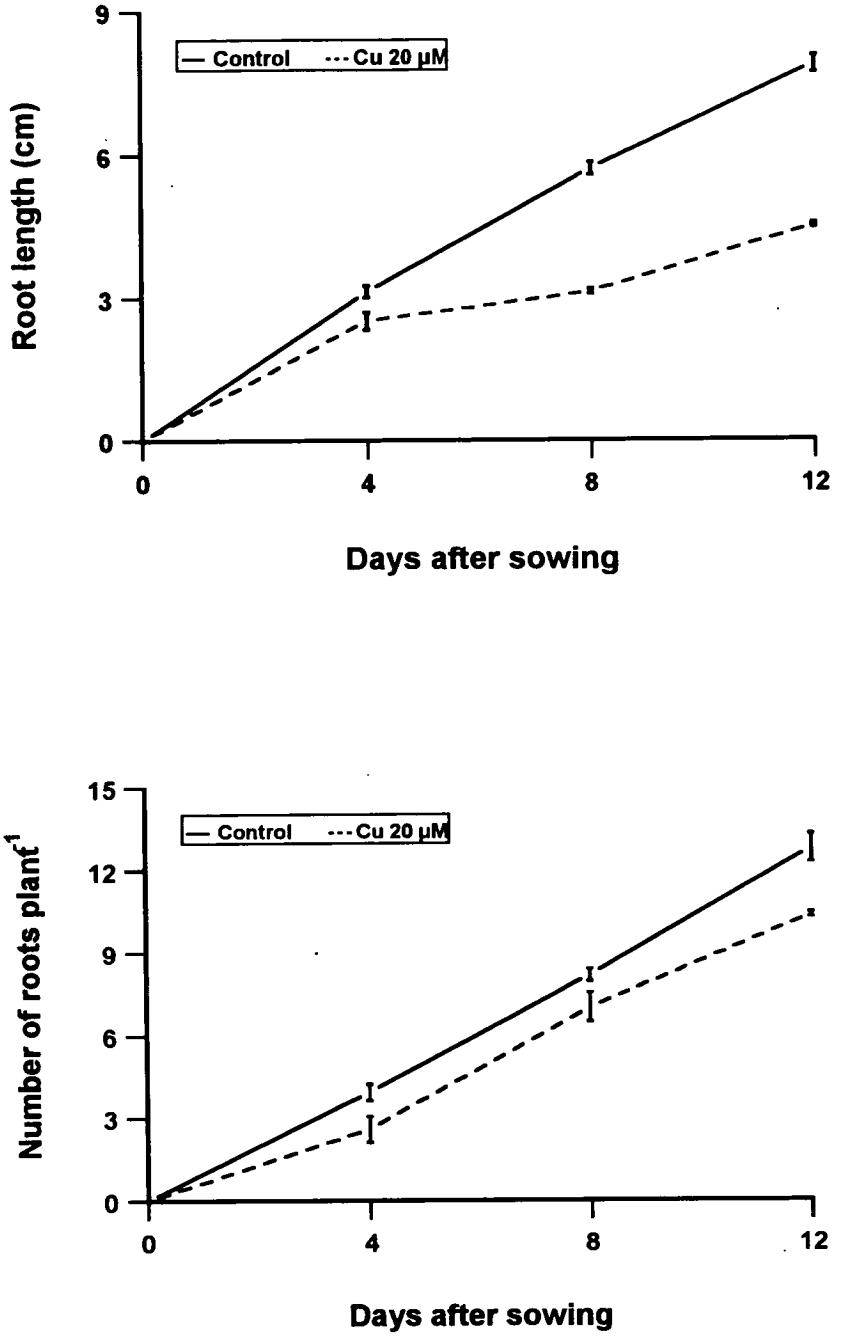


Fig. 5.1. Effect of copper ions on the length of the longest root and on the number of roots of seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

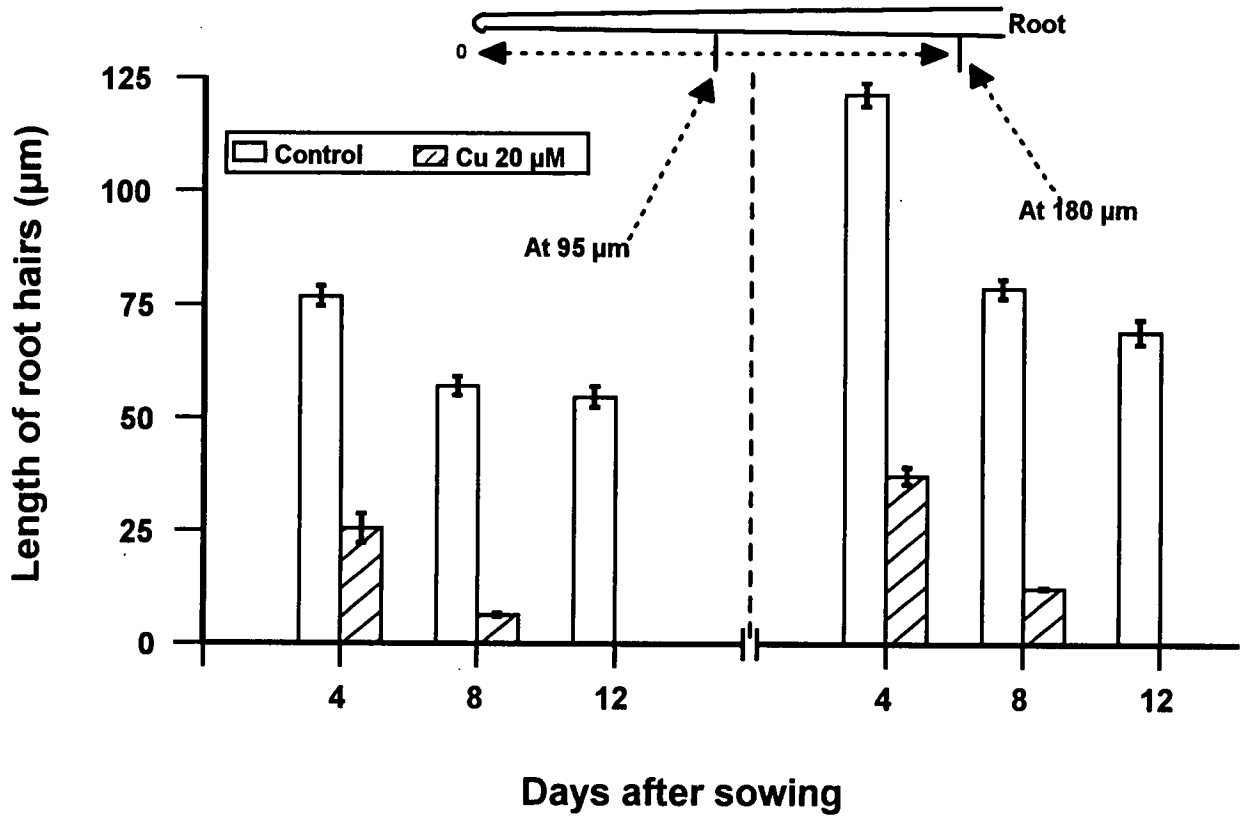


Fig. 5.2. Effect of copper ions on the length of root hairs at two distances from the root apex of seedlings of rice (*Oryza sativa* L) cv. NIAB. Error bar ± SEM, n=3.

The lengths of the root hairs were also measured at a distance of 180 μm from the apex of the longest root. In seedlings grown in the 20 μM copper ion solution for 4 d and 8 d the lengths of the root hairs were 70 % and 85 % less ($p < 0.01$) than those of the seedlings grown for the same period in the control solution. When the seedlings were grown in the control solution, the mean lengths of the root hairs after 8 d and 12 d were 35 % and 43 % less ($p < 0.05$) than the mean length of the root hairs of the seedlings after 4 d growth in the same solution. The difference between the mean length of the root hairs of the seedlings after 4 d and that of the seedlings after 8 d growth in the 20 μM copper ions solution was 66 % ($p < 0.01$), those measured after 8 d being shorter. However, there were no root hairs on the roots of the seedlings at this distance when the seedlings were grown in the 20 μM copper ion solution for 12 d. It was not only the length of the root hairs which was decreased but the density of the hairs was also decreased as a result of copper ion toxicity.

The mean distance from the apex to the start of the root hair-zone on the longest root of the seedlings after growth in the control and the 20 μM copper ion solution is presented in Fig. 5.3. The lengths of the hairless-zones on the roots of the seedlings after 4 d and 8 d growth in the 20 μM copper ions solution was 54 % and 70 % more ($p < 0.01$) than that of the seedlings grown in the control solution, respectively. The hairless-zone in the seedlings grown in the control solution was almost unchanged after 4, 8 and 12 d. However, in the seedlings grown in the 20 μM copper ions solution for 8 d it was 43 % more ($p < 0.05$) than that of the seedlings grown for 4 d and whole root was hairless after 12 d growth in the same solution.

The distance from the root apex to the point of emergence of the youngest lateral root on the longest root of 4, 8 and 12 d old seedlings grown in control solution and 20 μM copper ion solution is presented in Fig. 5.3. The distance of the point of emergence of the youngest lateral branch on the roots of the seedlings grown in the control solution for 8 d and 12 d was 96 % and 97 % less ($p < 0.01$) than that of the seedlings grown for 4 d in the same solution. The seedlings grown in the 20 μM copper ion solution for 4 dand 8 d had the youngest branch root emergence at a distance from the apex which was 99 % and 83 % less ($p < 0.01$) than that of the seedlings grown in the control solution for the same number of days. The seedlings grown in the 20 μM copper ion solution for 4 d had the youngest branch root behind

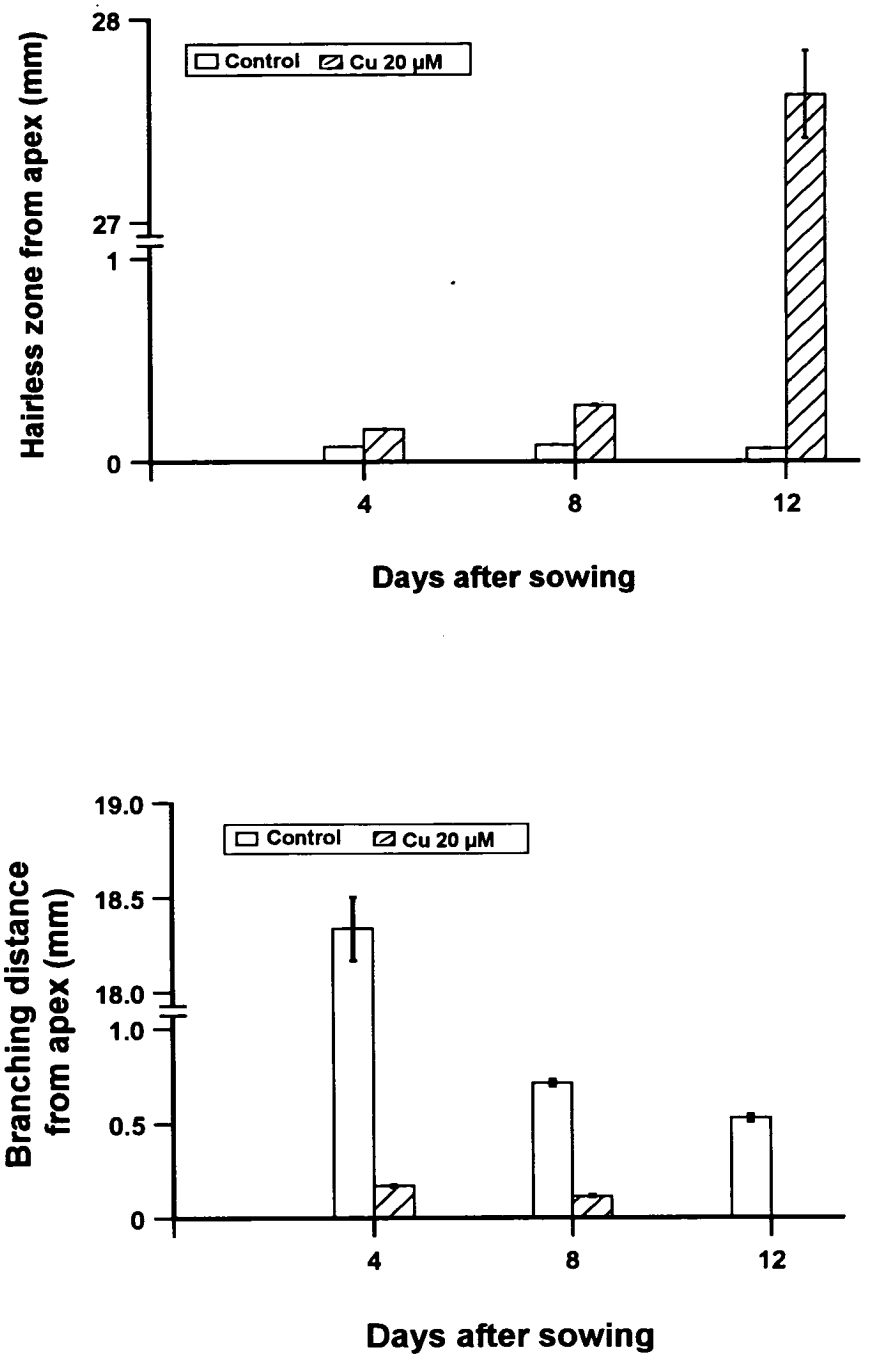


Fig. 5.3. Effect of copper ions on the hairless-zone and branching distance from the root apex of seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

the root apex appearing at a distance similar to that at which the youngest branch root appeared in the seedlings which were grown in the same solution for 8 d. However, root branching was observed at the apex when the seedlings were grown in the 20 μM copper ion solution for 12 d.

Plate 5.1 and 5.2 shows the scanning electron micrographs of the root tips of replica and of the seedlings which were cryopreserved. The root tip in both the control and the 20 μM copper ion treated seedlings was covered by a thick slimy material composed of mucilage and sloughed cells, and so it was not possible to see the cells of the root epidermis. In addition to that, a thick mass of root hairs was present around the root epidermis of the control seedlings thus making any observation of the morphology of the cells of the root epidermis difficult (Plate 5.3). In contrast, the scanning electron micrographs of the replicas of the roots (Plat 5.5) showed the cells of the epidermis clearly except where the tissue was damaged by the toxic effect of copper ions.

The longitudinal section of the roots showed that a thicker layer of root cap cells was present in the roots grown in 20 μM copper ion solution than in the control roots, and that the zone of cell elongation was almost absent in the copper-treated roots whereas it was well developed in the control roots (Plates 5.4). The meristematic zone in the roots of the seedlings grown in 20 μM copper ion solution was about 75 % smaller ($p < 0.01$) than that of the control seedlings. The cells in the tip of the control roots were square in longitudinal section or cuboidal in shape and uniform in size, and appeared to be undifferentiated up to a distance of about 0.2 mm from the tip. Further back from the apex, they are elongated in the long axis of the root, and back from that they are differentiated into the vascular cells (Plate 5.4). No lateral primordia were observed in the sections of the tip of the roots from the control seedlings.

When the seedlings were grown in the 20 μM copper ion solution for 4 d and 8 d, the length of the cells of the root epidermis at a distance of 0.5-0.6 mm from the apex was 35 % less ($p < 0.05$) and 60 % less ($p < 0.01$) than that of the seedlings grown in the control solution for the same periods (Fig. 5.4). The lengths of the cells of the root epidermis of the control seedlings after 8 d and 12 d were 22 % and 10 % more ($p < 0.05$) than the length of the epidermal cells of the control seedlings after 4 d growth (Plate 5.5 a). In contrast, the length of the cells of the root epidermis of the seedlings grown in the 20 μM copper ions solution for 8 d was 23 % less

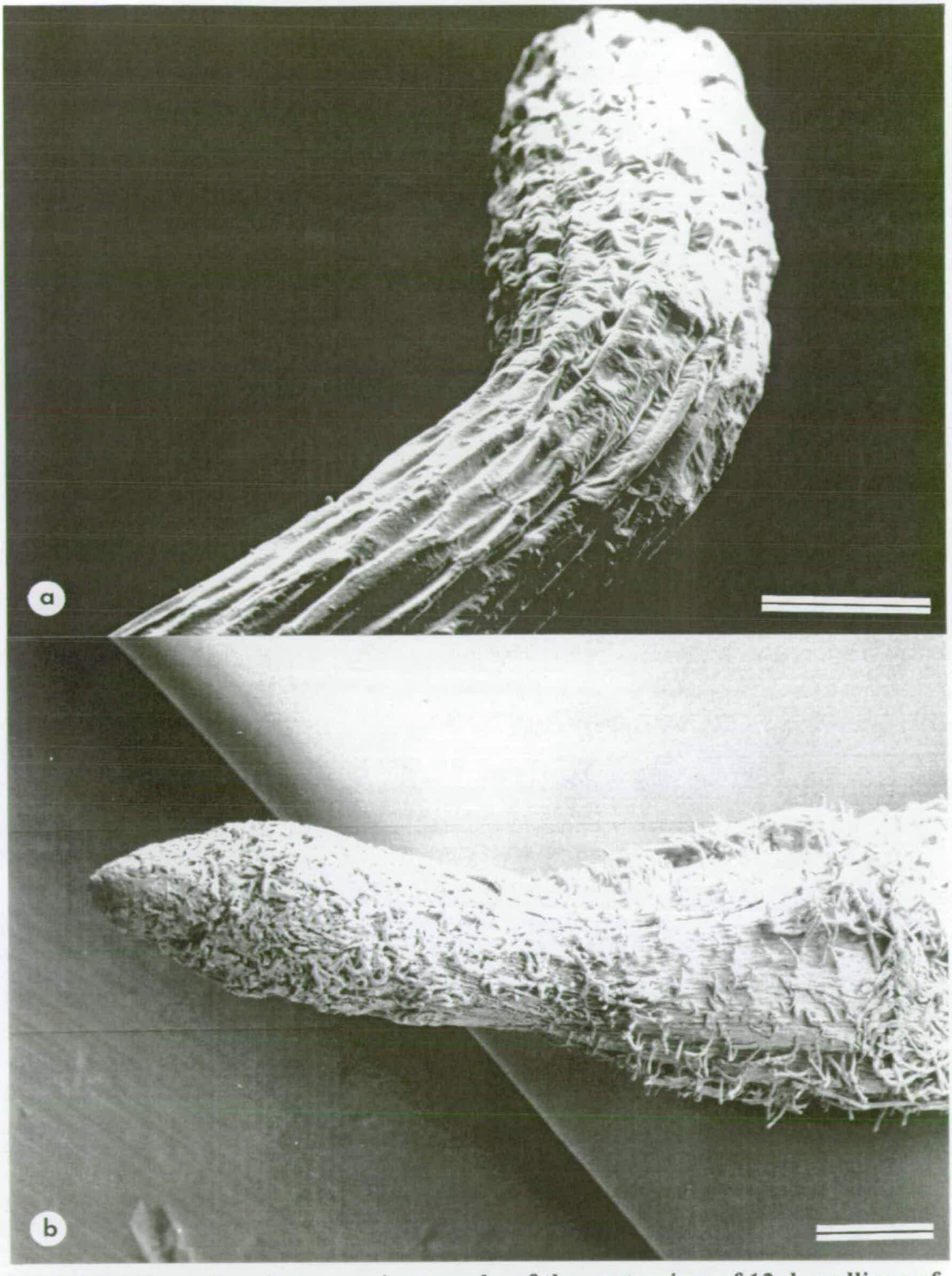


Plate 5.1. Scanning electron micrographs of the root apices of 12 d seedlings of rice grown in the Yoshida nutrient solution (control). (a) Replica. Scale bar = 40μm. (b) Cryopreserved. Scale bar = 200μm.

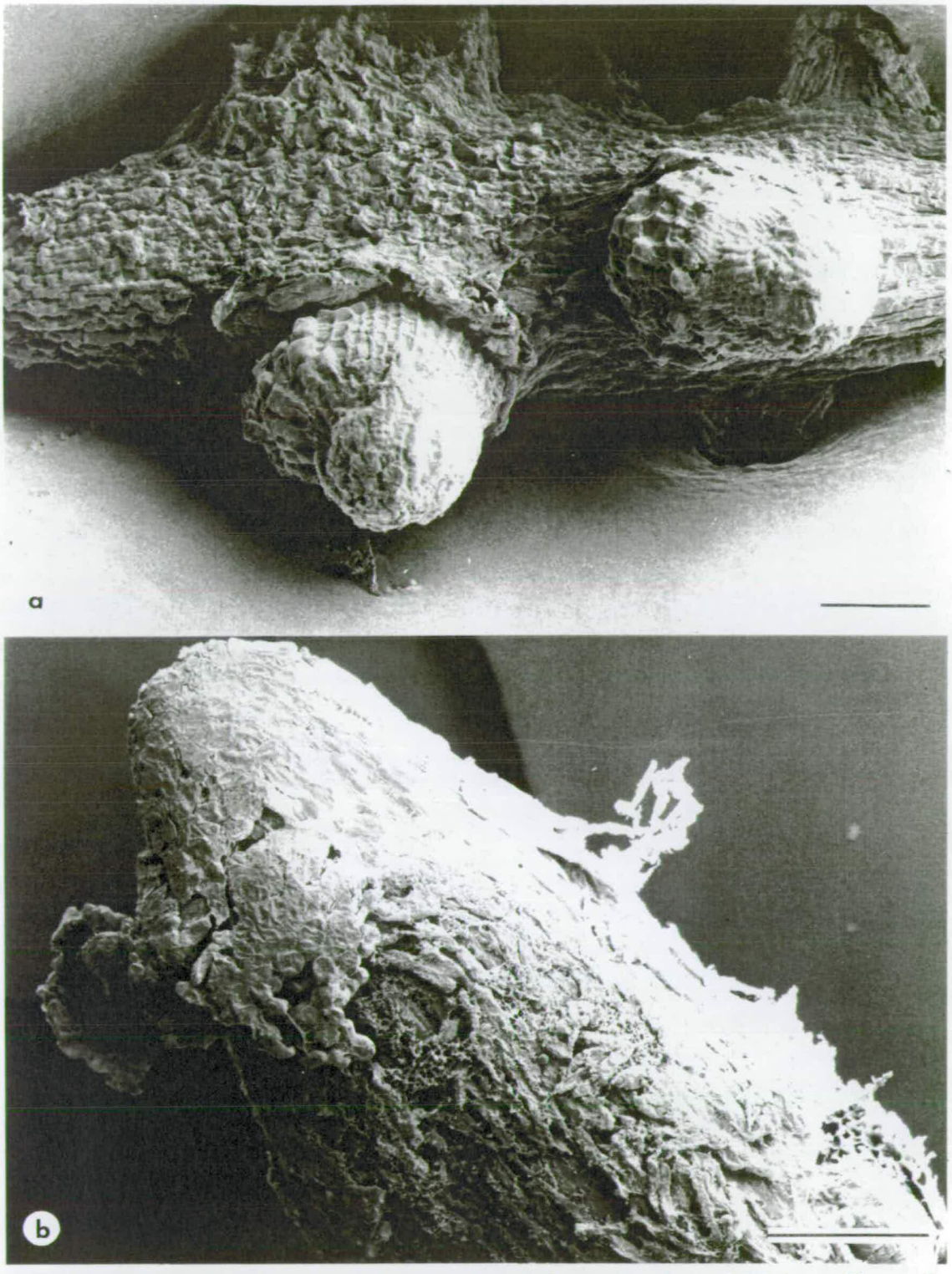


Plate 5.2. Scanning electron micrographs of the root apices of 12 d seedlings of rice grown in Yoshida nutrient solution containing 20μM copper. (a) Replica. Scale bar = 100μm. (b) Cryopreserved. Scale bar = 100μm.

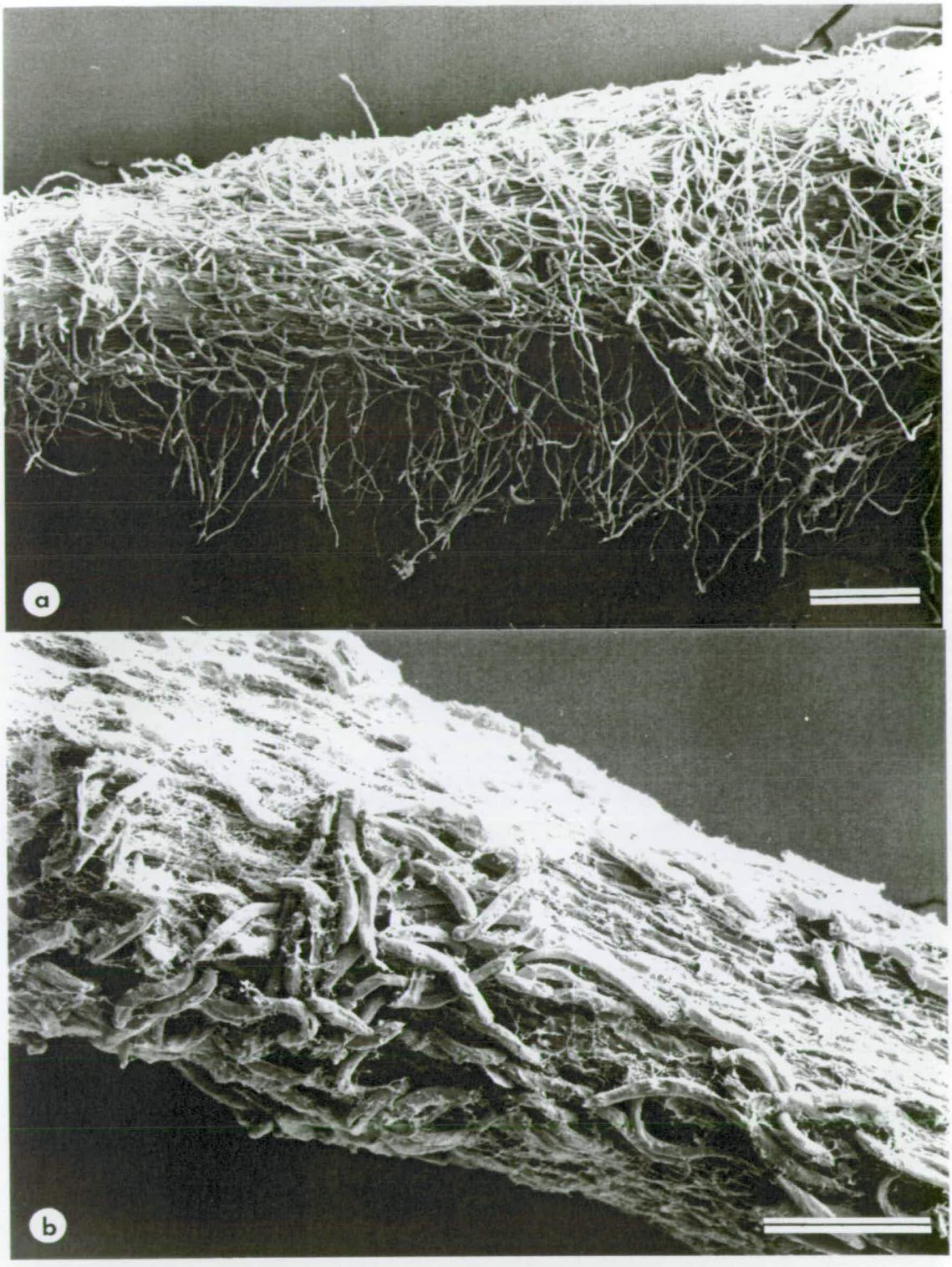


Plate 5.3. Scanning electron micrographs of the region behind the root apex of cryopreserved roots of 12 d seedlings of rice. (a) Seedlings grown in Yoshida nutrient solution (control) (adjoining Plate 5.1 b). Scale bar = 200μm. (b) Seedlings grown in Yoshida solution containing 20 μM copper. Scale bar = 100μm.

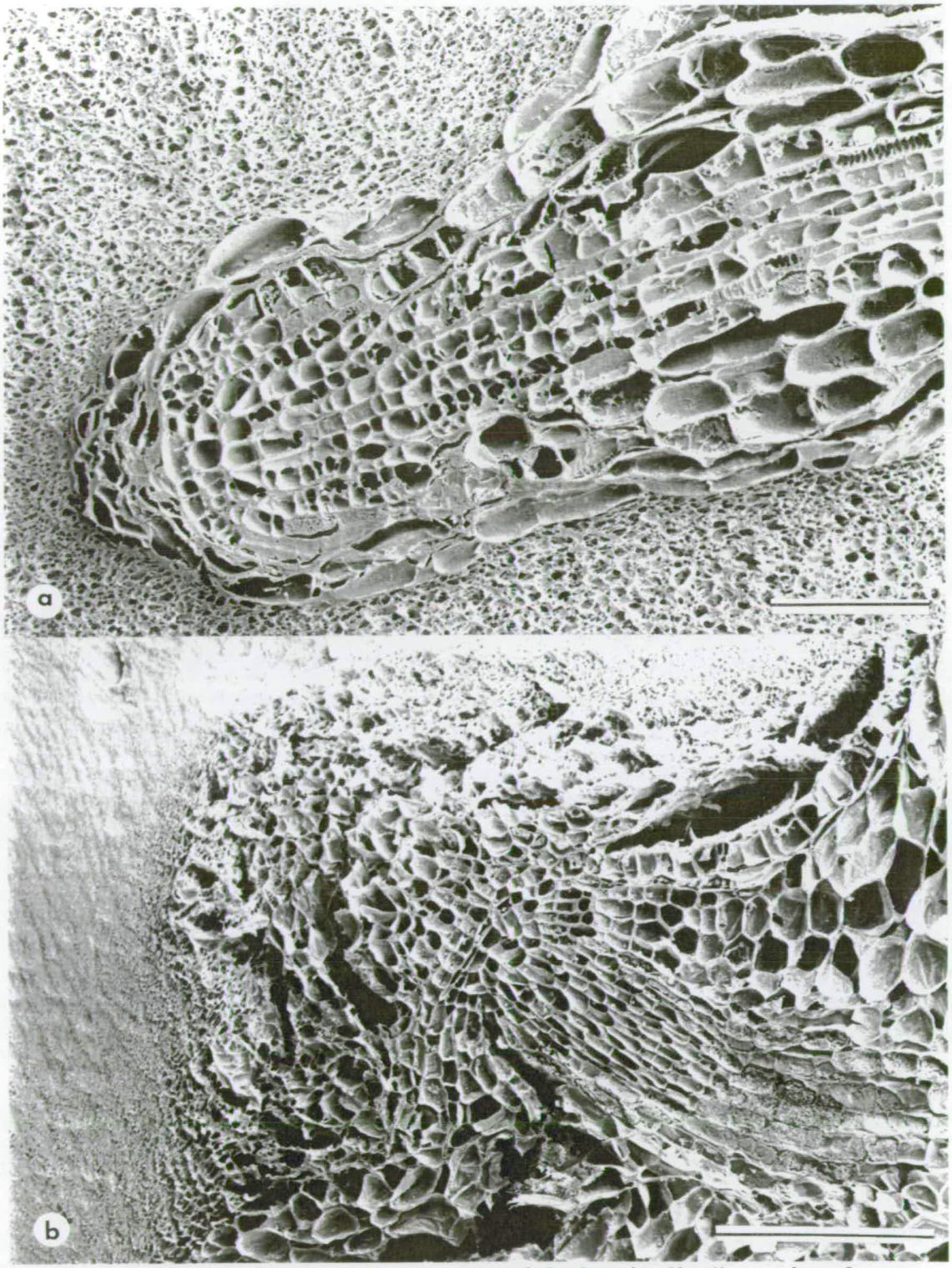


Plate 5.4. Scanning electron micrographs of the longitudinally sectioned roots of 12 d seedlings of rice. (a) Seedlings grown in Yoshida nutrient solution (control). Scale bar = 40μm. (b) Seedlings grown in Yoshida nutrient solution containing 20 μM copper. Scale bar = 100μm.

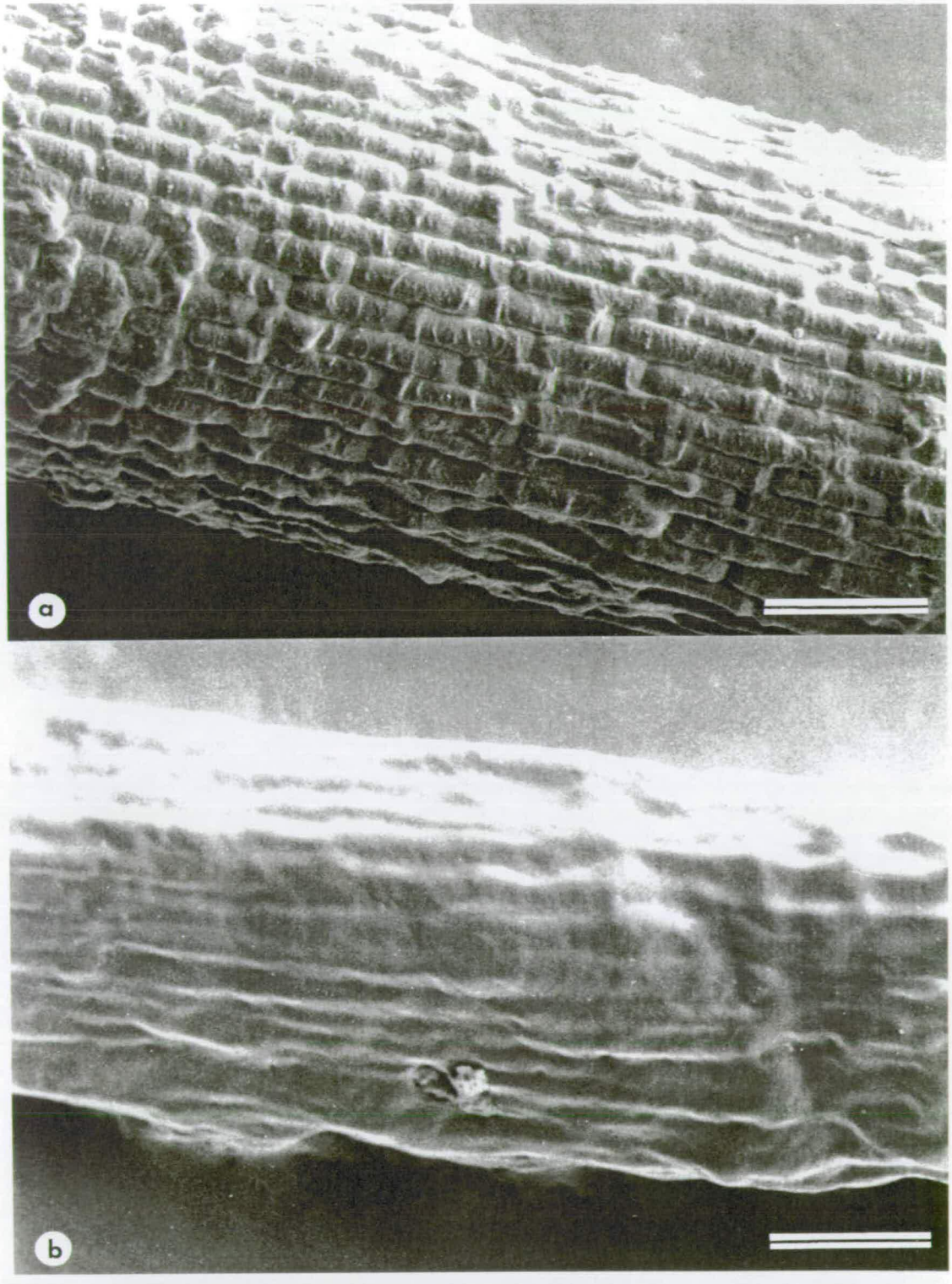


Plate 5.5. Scanning electron micrographs of replicas of the root surface 0.5 to 0.6 mm behind the root apex of 12 d seedlings of rice. (a) Seedlings grown in Yoshida nutrient solution (control). Scale bar = 40 μm. (b) Seedlings grown in Yoshida nutrient solution containing 20 μM copper. Scale bar = 40μm.

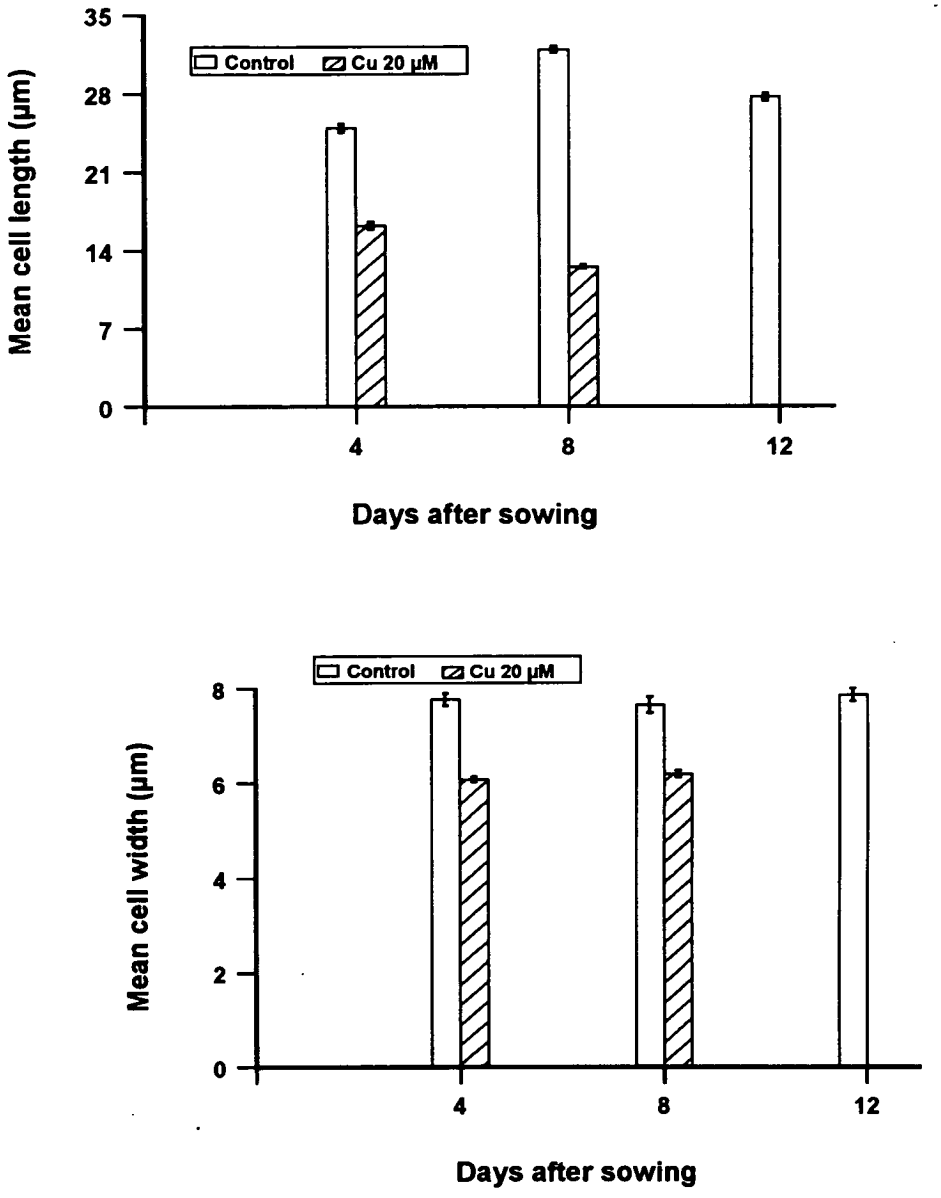


Fig. 5.4. Effect of copper ions on the length and on the width of the epidermal cells of the root between a distance of 0.5-0.6 mm from the apex of seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

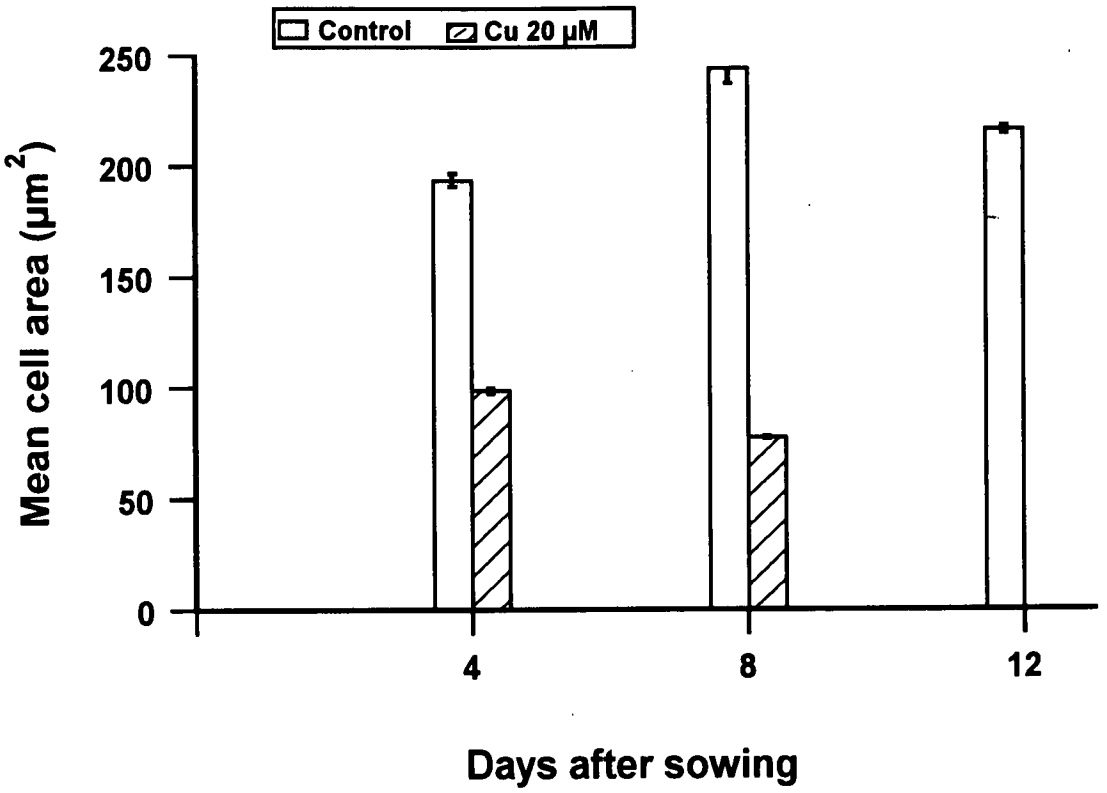


Fig. 5.5. Effect of copper ions on the mean area of the cells present in one template of root epidermis between 0.5 and 0.6 mm distance from the root apex of seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

($p < 0.05$) than that of the seedlings grown in the same solution for 4 d. It was not possible to measure the length of the cells of the root epidermis when the seedlings were grown in the 20 μM copper ion solution for 12 d, because the surface of the root was so much damaged that it was impossible to distinguish between the individual cells (Plate 5.5 b).

The widths of the cells of the root epidermis of the seedlings grown in the 20 μM copper ion solution for 4 and 8 d were 22 % and 19 % less ($p < 0.05$) than the cell width of the epidermal cells of the seedlings grown in the control solution for the same periods. However, the widths of the cells of the root epidermis after 4 d and 8 d were the same as when the seedlings were grown in the control solution for 12 d. Similarly the widths of the cells in the seedlings grown in the 20 μM copper ion solution for 4 d were similar to the widths of the seedlings grown for 8 d in 20 μM copper ion solution.

Values for the area of the cells of the root epidermis, are presented in Fig. 5.5. The cell widths were relatively constant and so the values for cell area followed a pattern similar to that which was observed for the cell length. When the seedlings were grown in the 20 μM copper ion solution for 4 d and 8 d the area of the epidermal cells was 50 % and 68 % less ($p < 0.01$) than that of the seedlings grown in the control solution for the same period. The area of the cells of the root epidermis of the seedlings grown in the control solution for 8 d and 12 d was 21 % and 11 % more ($p < 0.05$) than the area of the epidermal cells of the seedlings grown for 4 d in the same solution. The area of the root epidermal cells of the seedlings grown in the 20 μM copper ions solution for 8 d was 22 % ($p < 0.05$) less than those grown for 4 d in the same solution.

5.4. Discussion

Copper ions markedly inhibited root elongation, whereas a linear increase in root length was observed in the seedlings grown in the control solution. The root length of seedlings grown in copper ion solution was similar to that of the control seedlings when measured on the fourth day of the germination. De Vos *et al.* (1991) measured root length in a copper-sensitive cultivar of *Silene cucubalus* seedlings after 3 d of growth in nutrient solution supplemented with different concentrations of copper ions. They found 50 % inhibition in the root length of the seedlings at 4 μM copper ion concentration. In the present study, a much higher concentration of

copper ions was used and although a slight inhibition in root length was observed in 4 d old seedlings of rice, significant inhibition in root elongation was only observed in older seedlings. A concentration of 4 μM copper ions did not show any inhibition in root elongation in 20 d old seedlings of rice as observed in Section 4.2. An extrapolation of the results of the effect of 16 μM copper ion solution on root elongation in Section 4.4 showed that the observed inhibition in the root elongation of the seedlings was similar to that which was found in the present study. There was no effect of copper ions on the number of roots after 4 d and 8 d of seedling growth, however after 12 d they were less compared to the control. No effect of copper ions on the number of roots was observed in 7 d old seedlings grown in rolled paper towels (Section 3.3.).

The length and density of root hairs increased with distance away from the tip in seedlings grown in the control solution. Exposure of roots to 20 μM copper ions not only effectively inhibited the elongation of the root hairs but also suppressed their formation and the effect became more pronounced with the prolongation of the copper ion treatment. These observations confirm the results of Lidon and Henriques (1992 a) in rice seedlings, and of Patterson and Olson (1983) in *Betula* seedlings. No root hairs were formed in roots grown in toxic levels of copper ions. A similar effect has been shown in roots of soybean seedlings to be due to aluminium ions (Brady *et al.* 1993) and in roots of radish seedlings due to lead ions (Lane and Martin 1980). Moreover, root hair formation took place at a distance further away from the root tip, as shown by the effect of copper ions on the length of hairless-zone from root apex. Indeed, after 12 d the whole root was found to be hairless, the root hairs previously present being dead by this time. Brady *et al.* (1993) also observed a decrease in the root hair-zone and patches of stunted root hairs within the root hair zone in soybean seedlings grown in 2 μM aluminium ion solution. However, in contrast to the effect of copper and aluminium ions, root hair formation closer to the root tip due to zinc toxicity was reported in seedlings of *Festuca rubra* (Powell *et al.* 1988), and the effect was found to be much more pronounced in the sensitive populations than in the tolerant populations. Johnson-Flanagan and Owens (1985) and Jaunin (1988) have suggested that the formation of root hairs may extend nearer the tip if root growth is slowed down. The results of the present investigation appear to be contrary to this hypothesis, but it may be that the toxic effect of copper ions was so severe that it stopped emergence of the root hairs as well. It is generally accepted that root hair development and root hair turnover is a major means for establishing a well-developed

and efficient water and nutrient-absorbing root system. Root hair length is a prime factor in increasing nutrient uptake (Barber 1984). Therefore a decrease in the length of root hairs and lower root hair density due to copper ion toxicity is likely to have a negative effect on the capacity of the roots to absorb water and nutrients (Engenhart 1984).

Root elongation is inhibited by copper ions and so, after 12 d, lateral branching took place very close to the root tip. These lateral roots did not elongate. The total number of lateral branches per root was not counted so it could not be established whether copper ions induced the formation of lateral root primordia or whether the increased density of lateral root primordia was due to the inhibition of root elongation. However, observations on scanning electron micrographs show that in roots grown in copper solution there are few if any cells between neighbouring primordia, whereas in control roots there are many cells between neighbouring primordia. Davies *et al.* (1991) observed a stimulation in the number of lateral root primordia in zinc-tolerant and zinc-sensitive cultivars of *Festuca rubra* grown in 80 μM zinc. However, when both the cultivars were grown in 120 μM zinc there was a decrease in the number of lateral root primordia in the zinc-sensitive cultivars, though not in the zinc-tolerant. In normal growth, each lateral root appears at a relatively constant distance behind the tip of growing root and at a distance from the last lateral root as though some kind of positional information mechanisms were operating (Charlton 1982; Barlow and Adam 1988). Initiation of lateral root primordia occurs either in the root apex, as part of the general process of cell production, or it occurs in response to hormonal action (Charlton 1991). It has been suggested that one or more inhibitors of lateral root production, for example indole acetic acid, originate in the root apex (Torrey 1950, 1959; Wightman and Thimann 1980). Zeadan and Macleod (1984) suggested that inhibition in lateral root development in pea originates in the actively-proliferating cells of the apex of the primary root. It may be that inhibition in root growth due to copper ions is accompanied by a decrease in the amount of those inhibitors of lateral root development. However, the production and emergence of these lateral roots itself requires actively-dividing cells and some cell elongation and so it must be concluded that, at this level of copper treatment, some cell division continues in both the root apex and in the pericycle. At the point of emergence of the lateral root primordia, disruption of the endodermis and its casparian strip takes place. Dumbroff and Peirson (1971) suggested that there could be open apoplast pathways between cortex

and stele at these points. The presence of such a pathway has been shown in broad bean and maize by apoplastic tracer dyes, at the time when the lateral roots have just emerged from the parent root cortex (Peterson *et al.* 1981). In the presence of toxic levels of copper ions when root growth is severely inhibited and root hairs are stunted or absent, the emergence of more lateral roots leading to such an apoplastic pathway could be a part of a survival strategy followed by the plant. However, such an uptake would be accompanied by an increased influx of copper ions as well and any advantage to the plant would be short-lived.

Inspection of longitudinal sections of the roots showed that in copper ion-treated seedlings, the meristematic zone in the roots was shorter than that of the control seedlings and the zone of cell elongation was almost absent. A reduction in the length of the apical meristem of root in *Festuca rubra* due to zinc ion toxicity has been reported (Davies *et al.* 1991). Barlow and Adam (1989) detected a similar reduction in meristem length in roots of *Zea mays* grown at 5 °C compared with those grown at 20 °C. Decrease in the length of the meristem indicates that the effect of copper ions on roots changes the balance which normally exists between cell division, cell elongation and cell differentiation. Wainwright and Woolhouse (1975,1977) investigated effect of copper ions on root elongation in *Agrostis capillaris*. They reported that the first toxic effects of 1 μM copper ion are observed on root elongation rather than on root initiation. In another study, a copper ion concentration as low as 8 μM was toxic enough to kill the root meristem cells of a sensitive cultivar of the same species (Karataglis 1982). Apical meristems consist of initial cells and their proliferative derivatives. Barlow (1976) reported that the cells derived from the initials in a root meristem have a determinate period of reproductive capacity. For example, in *Allium cepa*, the cortical cells divide six to seven times, and then they start to elongate (González-Fernández *et al.* 1968). Copper ions may not completely inhibit the ability of cells to divide but may reduce the rate of cell division or the number of cells capable of dividing. A decrease in the size of the meristematic zone might result in a decreased production of inhibitory substances by the root meristem and thus stimulate the formation of lateral root primordia. Eventually, when lateral root primordia emerge from the epidermis they are exposed to the higher concentrations of copper ions and then fail to elongate. This gives rise to the star-shaped apex characteristic of the roots of seedlings grown in toxic concentrations of copper ions.

Dwivedi and Ahmad (1985) discussed the advantages of using fresh, hydrated plant material in scanning electron microscope. The fresh specimen gives a more accurate representation of the plant surfaces than conventional preparative techniques (Ledbetter 1976). However, the success of this technique is dependent on the mode of preparation to prevent dehydration and collapse. Dehydration and fixation has been recommended for scanning electron microscope studies. This technique avoids charging and the collapse of tissues during observation in the scanning electron microscope, but dehydration involves the use of organic solvents (ethanol, acetone, amylacetate), which dissolves the epicuticular waxes of specimens. Cryopreservation, the direct observation of frozen tissue, therefore may be considered the best available technology for observing delicate or difficult biological specimens. However, it was difficult to see the surface of cryopreserved roots clearly because mucilages attached to the surface and a thick blanket of root hairs completely obscured the epidermal cells. Echlin (1978) warned that low temperature techniques would not always be a reliable morphological tool, because there are many problems with specimen preparation, manipulation and transfer at low temperature together with an incomplete knowledge of the freeezing process in biological membranes, the redistribution of soluble constituents during freezing and the behaviour of frozen specimens during short and long-term storage at low temperature. The technique also involves high cost technology. The replica technique was found to be an accurate and durable alternative. It is rapid, non-destructive and gives good reproducibility (Williams *et al.* 1987). The main reason for using replicas of the roots for SEM was that they are simple to prepare, and provide a means of obtaining good images of difficult materials such as roots of seedlings which are covered by mucilages and root hairs, have a delicate surface and dehydrate rapidly. This method was used in order to determine the effect of copper ions on the length and width of the epidermal cells of the roots.

De Lima and Copeland (1994) made replicas of the roots of wheat seedlings grown in toxic concentrations of aluminium solutions to study the effect of aluminium ions on the root morphology. They reported damage to the epidermal cells and loss of cellular organisation. However, they did not measure the change in size of the epidermal cells. There are no studies about the effect of copper ions on the cell elongation in intact roots. This was made possible by using the replica technique described in the work reported here. The length of the epidermal cells of the roots of rice seedlings grown in 20 μM copper ions solution was significantly less than the

length of the epidermal cells of the control roots. The difference was greater the longer the root systems of the seedlings remained in the copper ion solution. The width of the epidermal cells of the roots grown in the copper ion solution was less than width of the epidermal cells of the control roots. Interestingly, it was unchanged in both treated and control seedlings sampled four days later. It is known that cells in the zone of elongation can elongate more than ten times their original cell length (Campbell 1993), however there was no mention in this paper of changes in the width of the cells. Cell elongation is a very complex process and it involves turgor requirements, loosening and synthesis of new cell walls and also growth regulators. Copper ions may cause inhibition of each or any of these factors responsible for cell growth, or they may have an indirect effect on a related aspect of cell metabolism (Wainwright and Woolhouse 1977).

6. MODIFICATIONS OF COPPER ION TOXICITY IN SEEDLINGS OF RICE *(Oryza sativa* L.*)* cv. NIAB 6.

6.1. Introduction

In order to have a better crop stand on soils which have been polluted by toxic metal ions, good agronomic practices and a means of ameliorating the toxic effects of the metals ions are required. As plants develop, their sensitivity to metal ions may change. Rice, which is transplanted at the seedling stage, may give a better crop stand if successfully protected from the toxic effects of copper ions at this stage. Different organic and inorganic ameliorants have been applied in order to protect plants from metal ion toxicity, especially aluminium toxicity. The role of these ameliorants in relation to the effects of copper ion toxicity in plants is not known. Thus, the extent to which these ameliorants are effective in protecting the plants, and the physiological basis of that protection need to be investigated.

Calcium and Magnesium

Amelioration of aluminium toxicity by calcium has been reviewed by Rengel (1992). There is evidence to suggest that calcium and magnesium ameliorate the effects of aluminium in wheat (Kinraid and Parker 1987; Keltjens and Dijkstra 1991) and sorghum (Tan *et al.* 1992 ab), of zinc in *Silene maritima* (Baker 1978), and of nickel (Robertson 1985) in maize seedlings. However, the role of calcium and magnesium in the amelioration of copper ion toxicity in plants has not been investigated. Edmeades *et al.* (1991) reported that both calcium and magnesium ameliorated the effects of aluminium toxicity in wheat seedlings, but plant growth was not restored to that of the control (i.e. no aluminium). They concluded that these two divalent cations have equal ameliorative effects. However, Kinraid and Parker (1987) found calcium more effective than magnesium in ameliorating the toxic effects of aluminium in wheat seedlings, and Keltjens and Dijkstra (1991) and Jan (1993) reported that magnesium was much more effective than calcium in protecting the roots of wheat seedlings against the deleterious effects of aluminium. The amelioration of deleterious effects of aluminium by calcium and/or magnesium is considered to be a direct physiological effect of the cations on aluminium toxicity (Edmeades *et al.* 1991) and is independent of the effects of increased ionic strength of these ions on chemical speciation of aluminium (Kinraid and Parker 1987).

Perhaps one of the most well-known functions of calcium in plants is its role in membrane stability and in the maintenance of cell integrity (Epstein 1972). In the

absence of calcium, the plasmamembrane becomes leaky and solutes are lost. Grant and Racz (1990) reported less leakage of solutes from roots of intact barley seedlings pre-treated with calcium and magnesium than from those pretreated with distilled water. They found that calcium and magnesium had similar effects on leakage through the plasmamembrane of intact roots. In contrast, Stevenink (1965) and Kawasaki *et al.* (1973), investigating the effect of divalent cations on the permeability of the plasmamembrane, found that magnesium was much less effective than calcium in reducing the leakage of solutes from discs of storage tissue of beetroot. Toxic metal ions which damage the plasmamembrane cause replacement of calcium ions from the plasmamembrane. This can be reversed by high concentrations of calcium ions at the plasmamembrane surface (Kirkby and Pilbeam 1984). Horst *et al.* (1991) reported that potassium leakage from the roots of soybean seedlings treated with aluminium was reduced when calcium was applied to them. Peroxidation of membrane lipids was reported in root tips of soybean due to aluminium toxicity and/or calcium deficiency (Cakmak and Horst 1991). However, there is no information about the effect of calcium and magnesium in relation to copper ion-induced lipid peroxidation and the leakage of potassium ions.

Citrate and Oxalate

Citrate and oxalate have been reported to reduce aluminium toxicity in ryegrass grown in nutrient solutions (Muchovej *et al.* 1988). Hue *et al.* (1986) studied the effect of organic acids on the amelioration of aluminium toxicity in cotton. They reported that the application of citrate and oxalate, at concentrations which are found in soils, greatly reduced the toxic effect of aluminium on tap-root growth. However, in contrast, Suthipradit *et al.* (1990) reported that oxalic acid failed to show any protective effect against aluminium toxicity in relation to tap-root elongation in soybean, cowpea and green gram. The role of citrate and oxalate amelioration of copper ion-toxicity has not been investigated. In addition, the effect of these organic acids on plasmamembrane permeability and lipid peroxidation is not known.

The present series of investigations was carried out in order to determine the role of calcium, magnesium, citrate and oxalate in modifying copper ion toxicity in relation to the growth of rice seedlings, and to monitor any changes induced by these substances on the effect of copper ions on plasmamembrane leakage and lipid peroxidation.

6.2. Calcium-induced modification of copper ion toxicity in seedlings of rice *(Oryza sativa* L.*)* cv. NIAB 6.

6.2.1. Experimental Procedure

A preliminary experiment was carried out to investigate the effect of calcium on root elongation of rice seedlings. The complete Yoshida nutrient solution contains 1 mM calcium which comes from $CaCl_2$. In order to achieve 1 (control), 4, 8, 12 and 16 mM calcium concentrations for the present experiment, a stock solution of 100 mM calcium was prepared using $CaCl_2$ and different volumes of this stock solution were incorporated into a minus-calcium buffered Yoshida solution to give the treatment concentrations. The procedure described in Section 2.2.1 was followed for the growth of the seedlings, except that the sheets of seeds were placed in specimen tubes containing buffered Yoshida nutrient solution pH 5.5, supplemented with, 1 (control), 4, 8, 12 and 16 mM $CaCl_2$. After 15 d the seedlings were removed from the specimen tubes, the length of the longest root was measured, and a dose-response curve was plotted (Fig. 6.1).

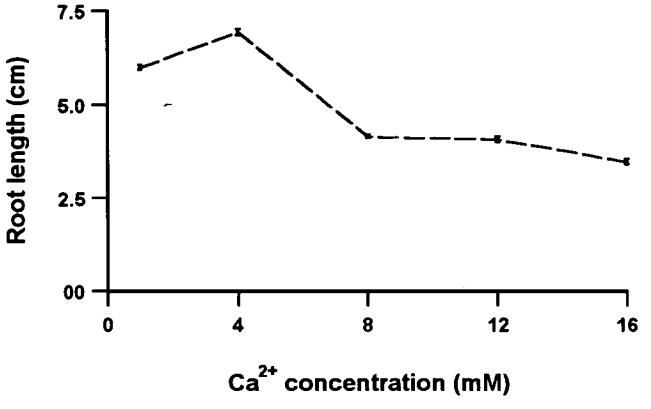


Fig. 6.1. Dose-response curve of the mean length of the longest roots of 15 d seedlings of rice (*Oryza sativa* L.) grown in Yoshida nutrient solution supplemented with different concentrations of calcium.

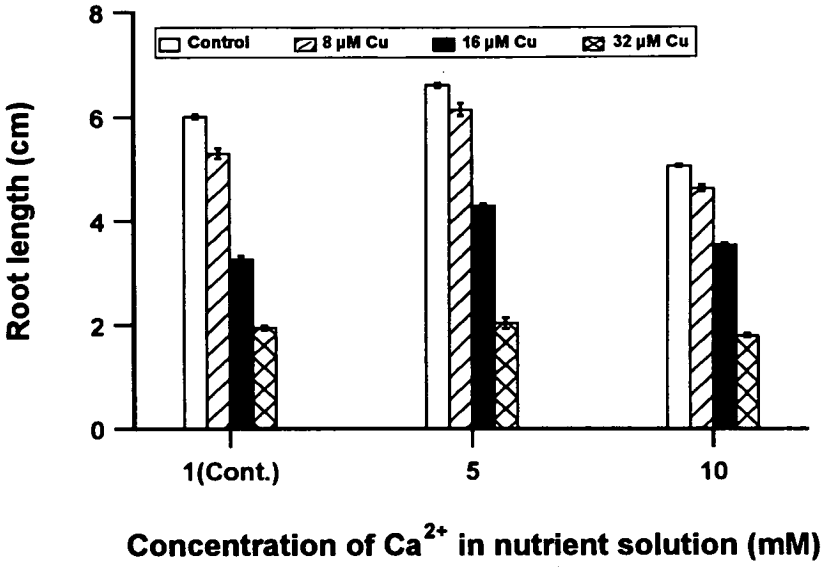
In the next experiment, the seedlings were grown in buffered Yoshida nutrient solution pH 5.5, containing 1 (control), 8, 16 and 32 µM $CuSO_4$, each at 1 (control), 5 and 10 mM $CaCl_2$. Growth conditions and experimental procedures

were the same as those described in Section 2.2.1. After 15 d, the length of the shoot and of the longest root, and the total root and shoot fresh and dry weights were measured following the method described in Section 2.2.1.

In order to determine the effect of calcium on copper ion-induced potassium leakage and on lipid peroxidation in roots, seedlings were grown for 15 d in the control nutrient solution. The whole root systems of intact plants were pre-incubated for potassium loading as described in Section 2.7. The seedlings were then divided into two sets. The root systems of set one were transferred to 100 ml of 0, (ultra-pure water), 1, 4, 8, 12 and 16 mM $CaCl_2$, and the root systems of set two were transferred to 100 ml of 0 (ultra-pure water, used as control), 5 mM and 10 mM $CaCl_2$ and 8 μM and 16 μM $CuSO_4$ alone, or in combinations, for 16 h. At the end of the 16 h period the concentration of potassium ions in the solution was measured by the procedure described in Section 2.7, and the TBA-rm content of the same roots was measured following the method described in Section 2.8.

6.2.2. Results

The effect of calcium on copper ion-induced toxicity in relation to the root and shoot length of the seedlings is presented in Fig. 6.2. The effect of different concentrations of copper ions on the root length of the seedlings grown in control (1 mM calcium) was similar to the effect of the same copper ion treatments on root length of the seedlings grown in control nutrient solution as presented in Section 4.4.4 (Fig. 4.10). The root lengths of the seedlings grown in the control, 8 and 16 μM copper ion solution at 5 mM calcium were 10 %, 16 % and 31 % more ($p < 0.05$) than the root lengths of the seedlings grown in the respective treatment solutions at 1 mM calcium. The root lengths of seedlings grown in the copper ion solutions at 10 mM calcium were smaller than the root lengths of the seedlings grown in the copper treatment solutions at 5 mM and 1 mM calcium. However, the percentage inhibition of root elongation in the seedlings grown in 8 μM, 16 μM and 32 μM copper ion solutions relative to the inhibition of root elongation in the seedlings in the respective control (1 μM copper) solution was less at 10 mM calcium than at 5 mM calcium. The copper-induced inhibition of shoot length was greater in the seedlings grown in buffered Yoshida solution containing 1 mM calcium than in the seedlings grown in the complete unbuffered Yoshida solution which also contains 1 mM calcium (Fig. 4.10). The inhibition in shoot length of the seedlings grown in 8 μM



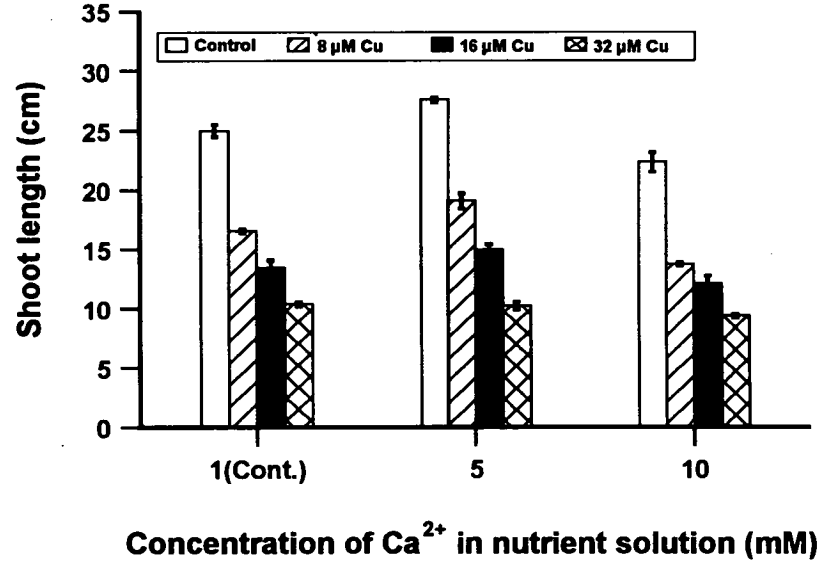


Fig. 6.2. Calcium-induced modification of the effects of copper ion toxicity on the length of the longest root and of the shoot of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

copper ion solution at 1 mM (cont.), 5 mM and 10 mM calcium levels was greater than the inhibition in the root length of the seedlings grown in the same treatment solutions. Shoot lengths in the seedlings grown in all treatment solutions were greater at 5 mM calcium than at 1 mM or 10 mM calcium. The treatment interaction was non-significant. However, the percentage inhibition in the shoot length of the seedlings grown in 16 μM and 32 μM copper ion solution relative to the control (1 μM copper) was less at 10 mM calcium than at 5 mM calcium.

The copper ion-induced inhibition of the root fresh weight of seedlings grown in Yoshida solution at different calcium levels followed a pattern similar to that of root length except at 5 mM calcium (Fig. 6.3). The root fresh weight of the seedlings grown in the copper ion solutions at 5 mM calcium was slightly less than the root fresh weight of the seedlings grown in the respective copper ion solutions at 1 mM calcium. The percentage difference between the root fresh weight of the seedlings grown in 32 μM and 16 μM copper was less than the percentage difference between the root lengths of the seedlings grown at the same treatment levels. The percentage inhibition in root fresh weight of the seedlings grown in 16 μM and 32 μM copper ion solution relative to the control (1 μM copper) was less at 10 mM calcium that at 5 mM calcium. When the seedlings were grown in 8 μM copper ion solution at 5 mM calcium the percentage inhibition in shoot fresh weight was greater than the percentage inhibition in the shoot length of the seedlings grown in the same treatments. The seedlings grown in 1 μM copper (cont.) at 5 mM calcium showed maximum shoot fresh weight whereas minimum shoot fresh weight was observed when the seedlings were grown in 32 μM copper ion solution at the same calcium level. The percentage inhibition in the shoot fresh weight of the seedlings grown in 8 μM, 16 μM and 32 μM copper ion solution relative to the control (1 μM copper) was less at 10 mM calcium that at 5 mM calcium.

The results presented in Fig. 6.4. shows the effect of calcium-induced modification of copper ion toxicity in relation to the root and shoot dry weight of the seedlings. The root dry weight of the seedlings grown in the copper ion solutions at 1 mM calcium (cont.) and 10 mM calcium followed a pattern similar to that which was observed for root length and root fresh weight. When the seedlings were grown in 16 μM copper ion solution, the root dry weight of the seedlings at 5 mM calcium was 49 % more ($p < 0.05$) than the root dry weight of the seedlings in the same copper ion treatment at 1 mM calcium. The percentage inhibition in the root dry

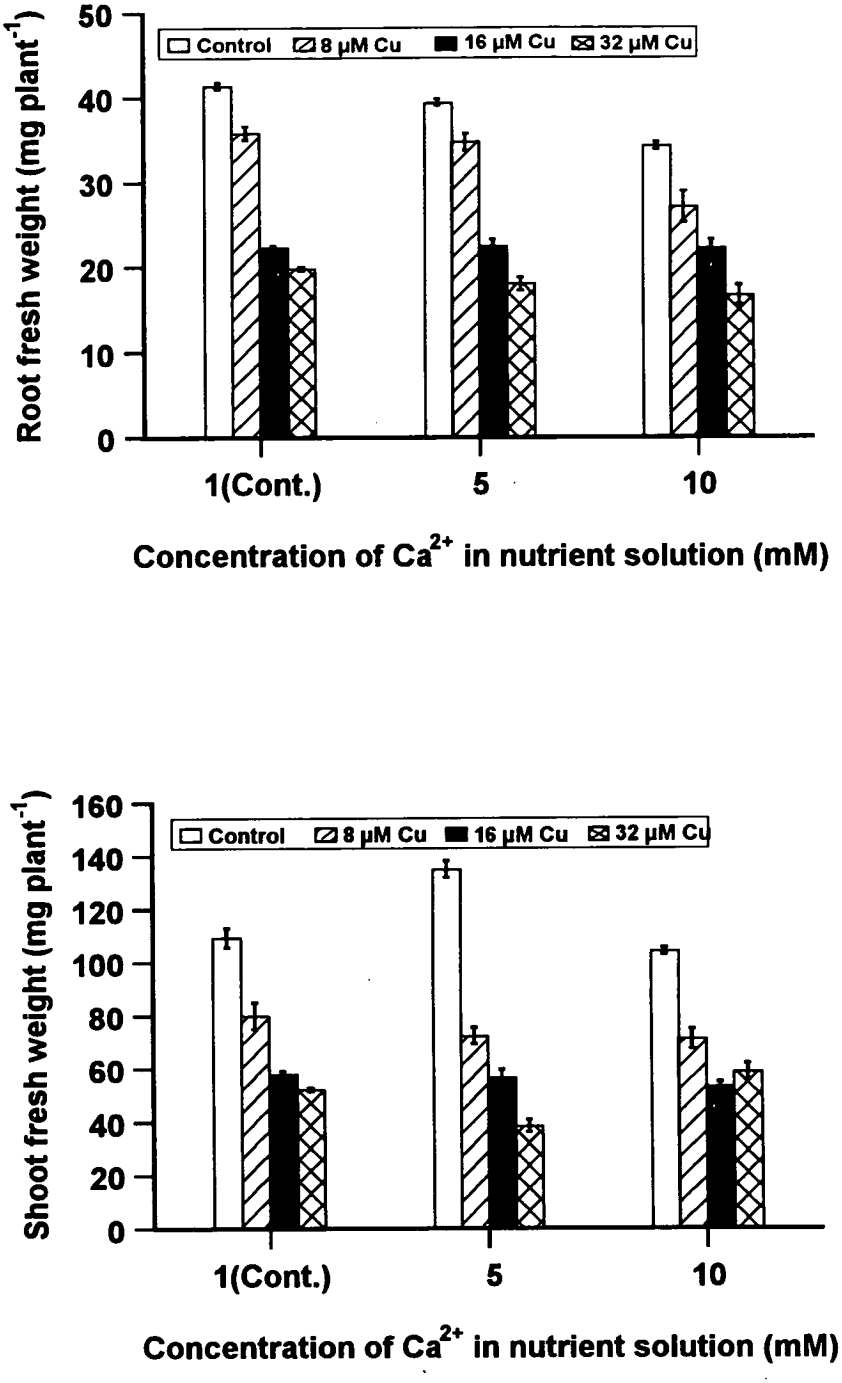


Fig. 6.3. Calcium-induced modification of the effects of copper ion toxicity on root and shoot fresh weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

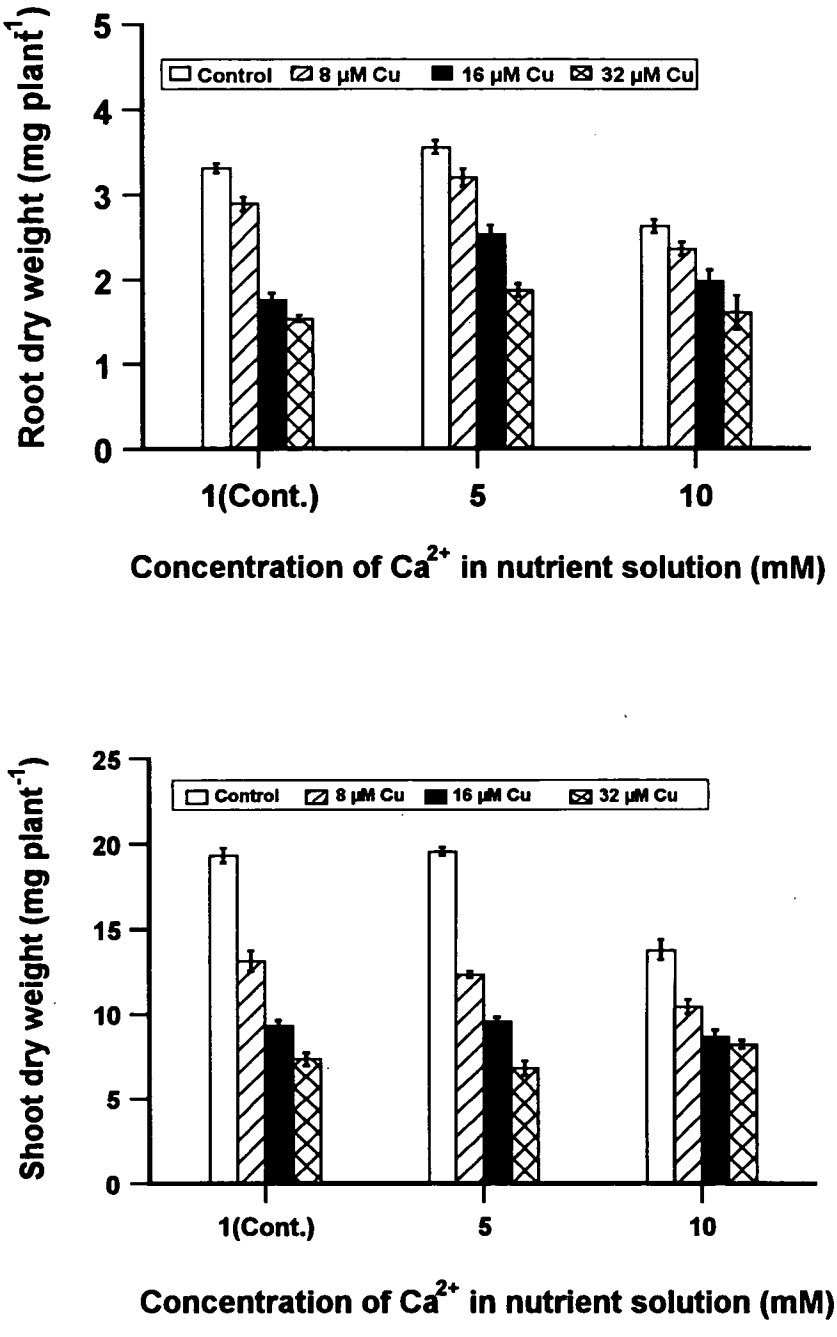


Fig. 6.4. Calcium-induced modification of the effects of copper ion toxicity on root and shoot dry weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

weight of the seedlings grown in 8 μM, 16 μM and 32 μM copper ion solutions relative to the respective control was smaller at 10 mM calcium than at 5 mM calcium. Shoot dry weight of the seedlings grown in the control solution (1μM copper) at 10 mM calcium was 28 % less ($p < 0.05$) than the shoot dry weight of the seedlings grown in the control solution (1 μM copper) at 1 mM calcium, whereas the shoot fresh weight of the seedlings grown in the control solution (1 μM copper) at 10 mM calcium was not significantly different from that of the seedlings grown in the control (1 μM copper) solution at 1 mM calcium. Shoot dry weight of the seedlings grown in the control solution (1μM copper) at 5 mM calcium was the same as that of the seedlings in the same copper solution at 1 mM calcium, however, the shoot fresh weight of the seedlings grown in same copper ion solution at 5 mM calcium was 24 % more ($p < 0.05$) than that of the control (1 mM calcium). The percentage inhibition in the shoot dry weight of seedlings grown in 8 μM, 16 μM and 32 μM copper ion solution relative to the respective control was smaller at 10 mM calcium than at 5 mM calcium.

The results obtained by using the computer simulation programme GEOCHEM-PC to investigate copper ion speciation at various calcium and copper ion levels in the buffered nutrient solution are presented in Fig. 6.5. Detailed results in relation to all the nutrients are presented in Appendix IV. When 1 (cont.), 8 μM and 16 μM copper were present in addition to 1 mM (cont.), 5 mM and 10 mM calcium levels, less than 0.25 % of the copper was present as the free metal ion. The remaining copper present was complexed with EDTA, and no precipitation of copper was predicted. When 32 μM copper was present in addition to calcium at each of the 3 calcium concentrations, it was found that less than 1 % copper was present as free metal ions, about 99 % was complexed with EDTA, and no precipitation of copper was predicted. Results obtained for calcium speciation (Fig. 6.5.) in the above treatment solutions showed that for each calcium level more than 80 % of added calcium was present as the free metal ion at all the copper ion levels.

The effect of calcium ions on potassium leakage and on the amount of TBA-rm in the whole intact root systems of the seedlings is presented in Fig. 6.6. The amount of potassium ions detected in the 4 mM and 12 mM calcium solution after incubation of the whole intact root systems of the seedlings was 47 % and 77 % less ($p < 0.01$) than that measured in 1 mM calcium solution. The amount of TBA-rm

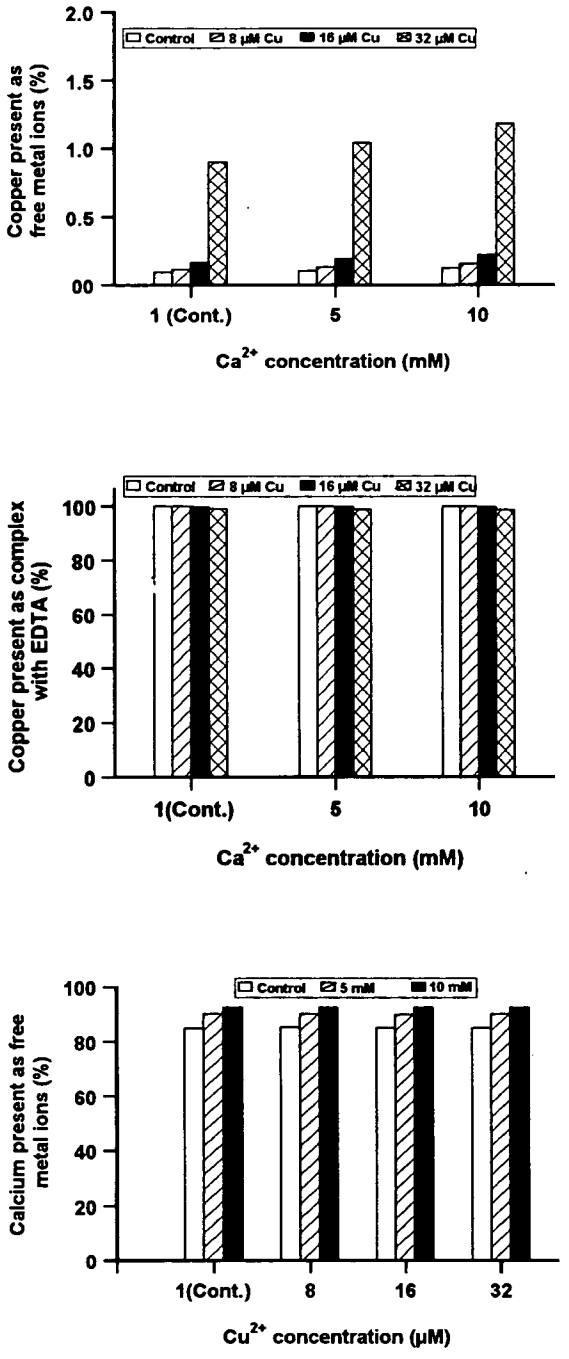


Fig. 6.5. Percentage of added copper and calcium present in ionic form, and of copper complexed with EDTA in the complete Yoshida nutrient solution at different calcium levels as predicted by GEOCHEM-PC.

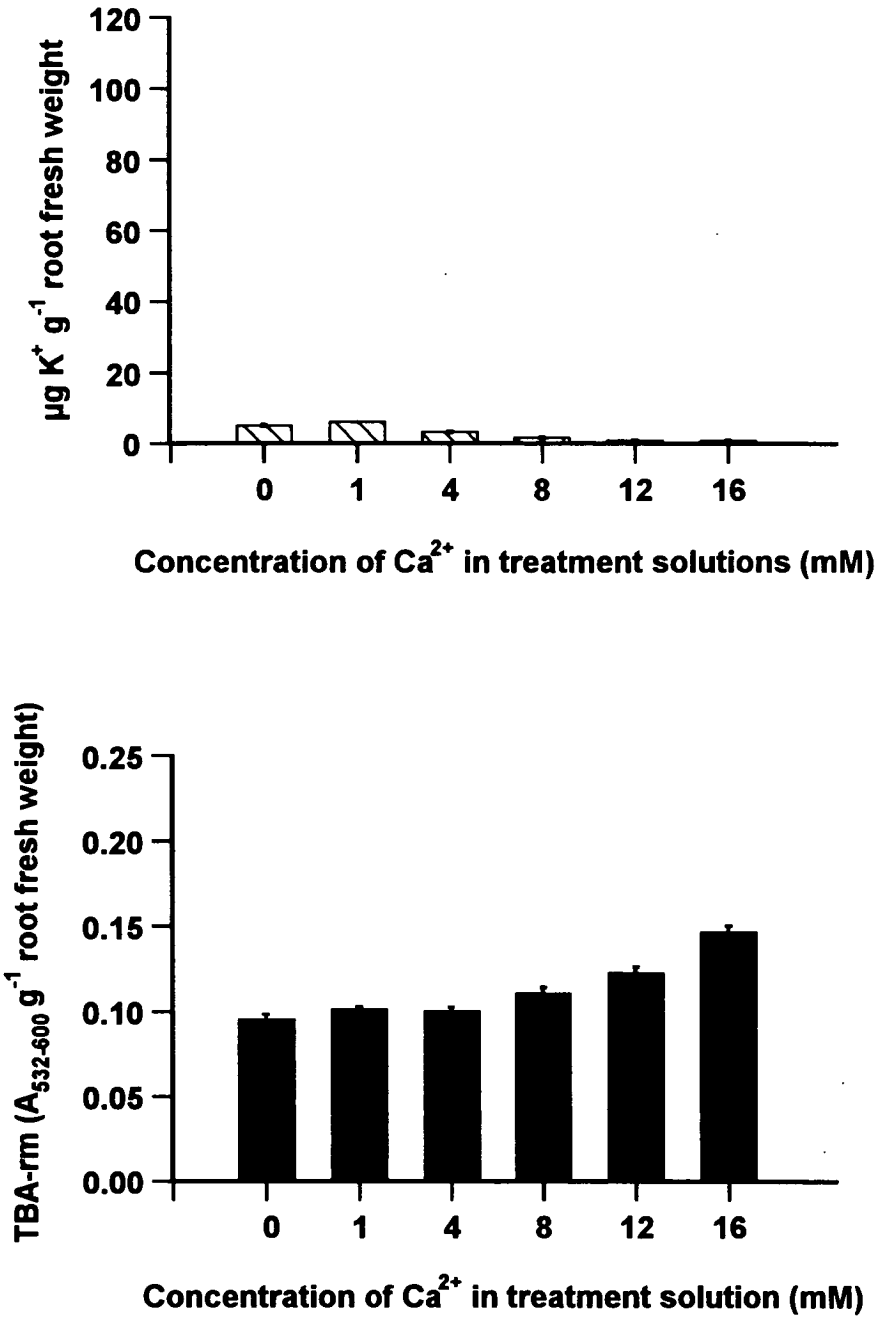


Fig. 6.6. Effect of different concentrations of calcium ions on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

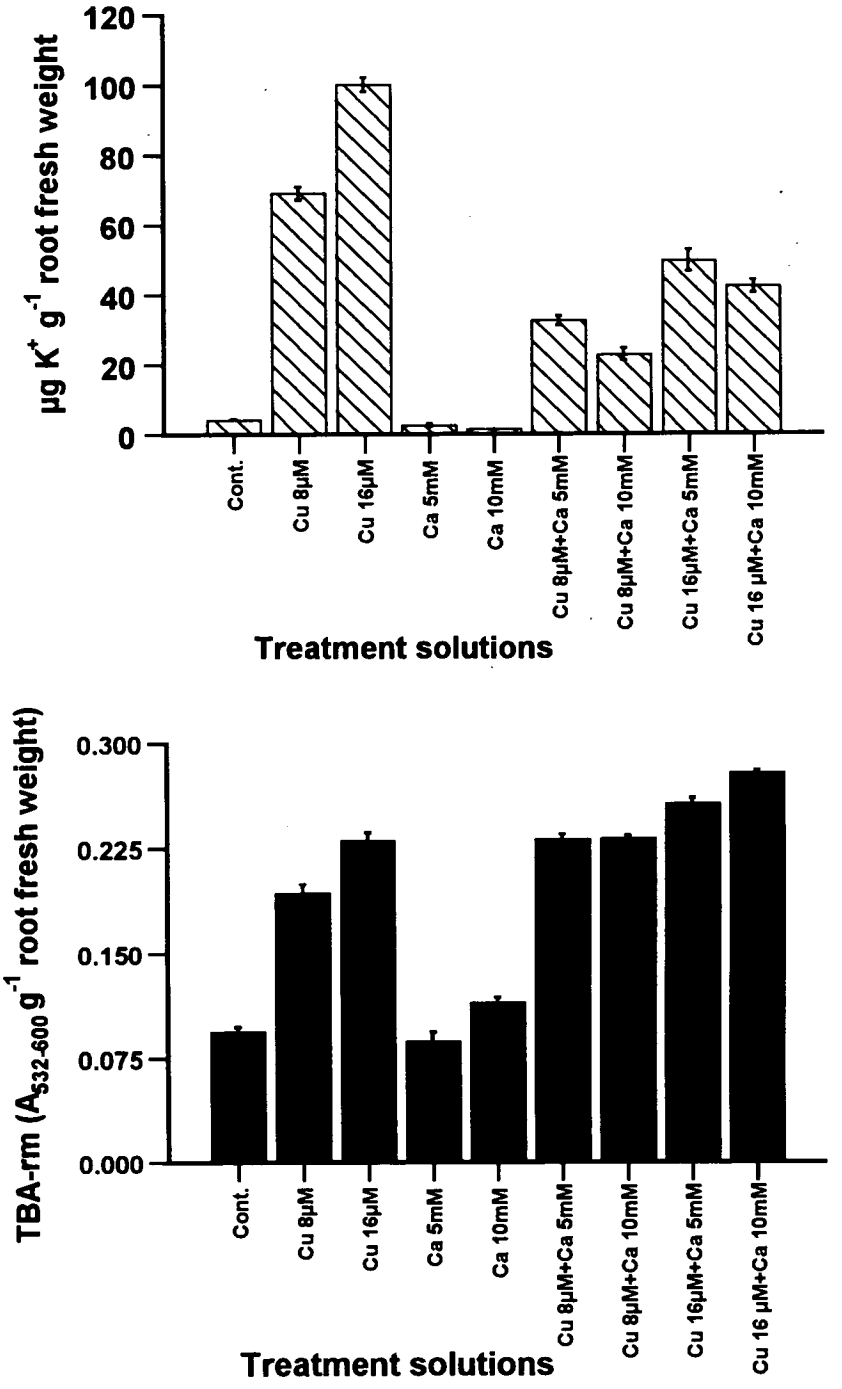


Fig. 6.7. Calcium-induced modification of the effects of copper ion toxicity on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

accumulated in the roots of the seedlings after incubation with 4 mM calcium was slightly less than that accumulated in the roots after they had been incubated in 1 mM calcium. The amount of TBA-rm accumulated in the roots after incubation with 12 mM calcium was 30 % more ($p < 0.05$) than the amount of TBA-rm accumulated in the roots of the seedlings after they had been incubated in the 1 mM calcium treatment solution.

The calcium-induced modification in the amount of potassium ion leakage and TBA-rm accumulation in relation to copper ion toxicity in the whole intact root systems of the seedlings is presented in Fig. 6.7. The amount of potassium ions leaked from seedlings incubated in the 8 μM copper + 5 mM calcium and 8 μM copper + 10 mM calcium solutions was 53 % and 67 % less ($p < 0.01$) than the amount of potassium ions leaked from seedlings incubated in the 8 μM copper solution. The amount of potassium ions leaked from seedlings incubated in 16 μM copper + 5 mM calcium and 16 μM copper + 10 mM calcium solutions was 50 % and 58 % less ($p < 0.05$) than the amount of potassium ions leaked from seedlings incubated in the 16 μM copper solution. The amount of potassium leaked into the 8 μM copper + 10 mM calcium solution was 15 % ($p < 0.05$) less than the amount of potassium leaked into the 8 μM copper + 5 mM calcium solution. However, the amount of potassium ions leaked into the 16 μM copper + 10 mM calcium solution was slightly higher than the amount of potassium ions leaked into the 16 μM copper + 5 mM calcium solution.

The amount of TBA-rm in the roots after incubation with 8 μM copper + 5 mM calcium and with 8 μM copper + 10 mM calcium was in both cases 20 % more ($p < 0.05$) than the amount of TBA-rm in roots which had been incubated in 8 μM copper. When the roots were incubated in the 16 μM copper + 5 mM calcium and 16 μM copper + 10 mM calcium solutions, the amount of TBA-rm was 9 % and 21 % more ($p < 0.05$) than that of the roots of the seedlings incubated in 16 μM copper. When the roots were incubated in the 16 μM copper + 10 mM calcium solution the amount of TBA-rm was 9 % more ($p < 0.05$) than that in the roots when they were incubated in the 16 μM copper + 5 mM calcium solution.

6.3. Magnesium-induced modification of copper ion toxicity in seedlings of rice *(Oryza sativa* L.*)* cv. NIAB 6.

6.3.1. Experimental Procedure

A preliminary experiment was carried out to investigate the effect of magnesium on root elongation of rice seedlings. The complete Yoshida nutrient solution contains 1.6 mM magnesium which comes from $MgSO_4$. In order to achieve 1, 2, 4, 6 and 8 mM magnesium concentrations for the present experiment, a stock solution of 100 mM magnesium was prepared using $MgCl_2$ and different volumes of this stock solution were incorporated into a minus-magnesium buffered Yoshida solution to give the treatment concentrations. In this experiment, therefore, 1 mM magnesium, instead of 1.6 mM magnesium was used as the control. The procedure described in Section 2.2.1 was followed for the growth of the seedlings, except that the sheets of seeds were placed in the specimen tubes containing buffered Yoshida nutrient solution pH 5.5, supplemented with, 1 (control), 2, 4, 6 and 8 mM $MgCl_2$. After 15 d the seedlings were removed from the specimen tubes, the length of the longest root was measured, and a dose-response curve was plotted (Fig. 6.8).

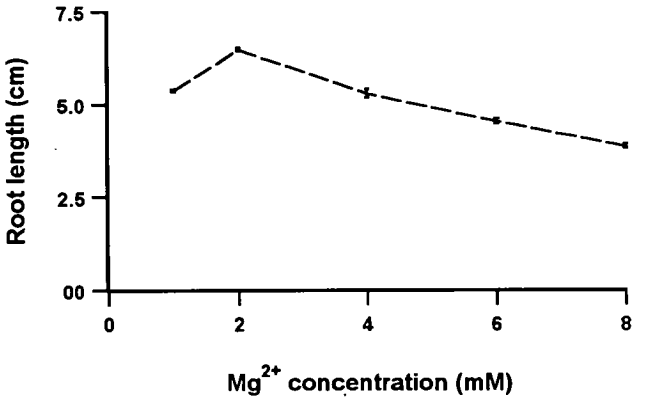


Fig. 6.8. Dose-response curve of the mean length of the longest roots of 15 d seedlings of rice (*Oryza sativa* L.) grown in Yoshida nutrient solution supplemented with different concentrations of magnesium.

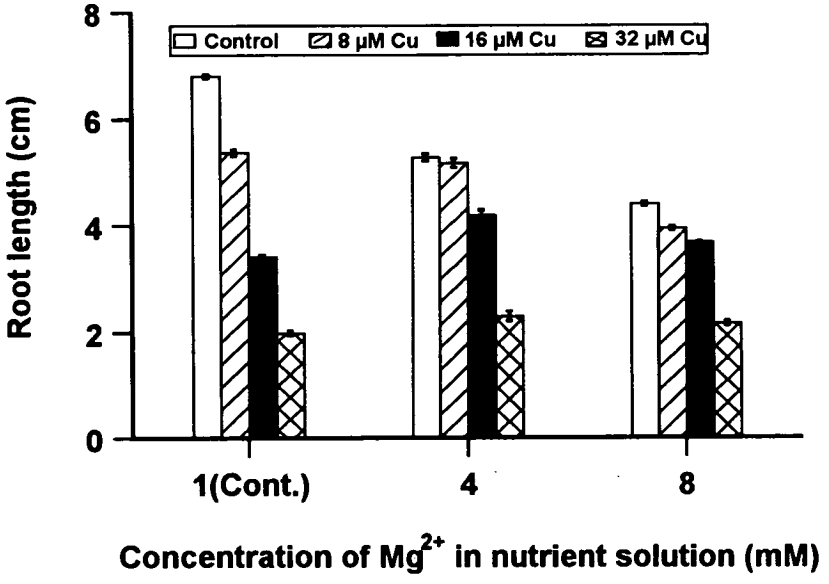
In the next experiment, the seedlings were grown in buffered Yoshida nutrient solution pH 5.5, containing 1 (control), 8, 16 and 32 µM $CuSO_4$, each at

1 (control), 4 and 8 mM $MgCl_2$. Growth conditions and experimental procedures were the same as those described in Section 2.2.1. After 15 d, the length of the shoot and of the longest root, and the total root and shoot fresh and dry weights were measured following the methods described in Section 2.2.1.

In order to determine the effect of magnesium on copper ion-induced potassium leakage and on lipid peroxidation in roots, seedlings were grown for 15 d in the control nutrient solution. The whole root systems of intact plants were pre-incubated for potassium loading as described in Section 2.7. The seedlings were then divided into two sets. The root systems of set one were transferred to 100 ml of 0 (ultra-pure water), 1, 2, 4, 6 and 8 mM $MgCl_2$ and the root systems of set two were transferred to 100 ml of 0 (ultra-pure water, used as control), 4 and 8 mM $MgCl_2$ and 8 and 16 μM $CuSO_4$ alone, and in combinations, for 16 h. At the end of the 16 h period the concentration of potassium ions in the solution was measured by the procedure described in Section 2.7, and the TBA-rm content of the same roots was measured following the method described in Section 2.8.

6.3.2. Results

Figure 6.9. shows the magnesium-induced modification of copper ion toxicity in relation to the mean length of the longest root and of the shoot of seedlings of rice grown in the treatment solutions. The treatment interaction was non-significant. The root lengths of the seedlings grown in Yoshida solutions supplemented with 4 mM and 8 mM magnesium ions were 23 % and 35 % less ($p < 0.05$) than those of the seedlings grown in the same solution at 1 mM magnesium. The root length of the seedlings grown in 8 μM copper ion solution at 4 mM magnesium was slightly less than the root length of the seedlings grown in respective control. The root length of the seedlings grown in 16 μM copper ion solution at 4 mM magnesium was 23 % longer ($p < 0.05$) than the root length of the seedlings grown in the same concentration of copper ions at 1 mM magnesium. The root lengths of seedlings grown in copper solutions at 8 mM magnesium were less than the root lengths of the seedlings grown in 4 mM or 1 mM magnesium. However, the percentage inhibition of root elongation in the seedlings grown in 16 μM and 32 μM copper solution relative to the inhibition in root elongation in the seedlings in the respective control (1μM copper) solution was less at 8 mM magnesium. The shoot length of the seedlings grown in the control



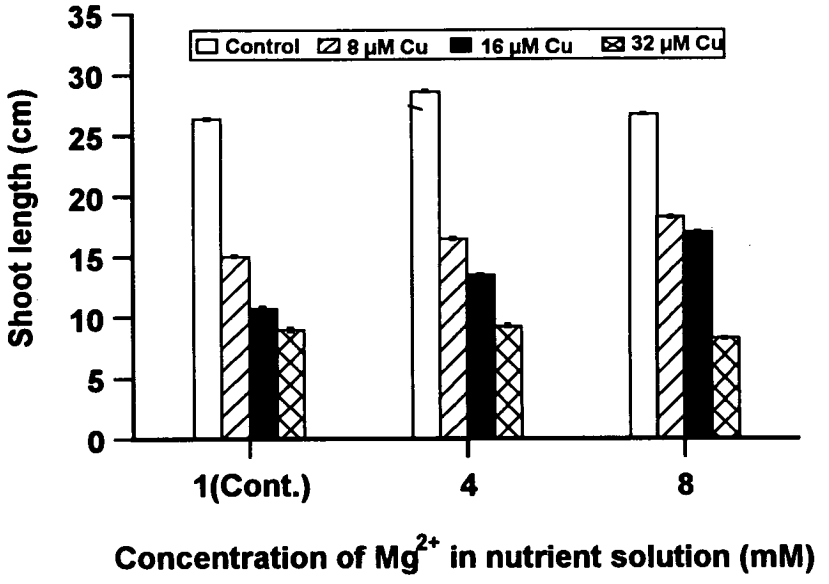


Fig. 6.9. Magnesium-induced modification of the effects of copper ion toxicity on the length of the longest root and of the shoot of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

(1 μM copper) solution at 4 mM magnesium was 9 % greater ($p < 0.05$) than the shoot length of the seedlings grown in the control solution at 1 mM magnesium. The inhibition in shoot length of the seedlings grown in 8 μM copper solution at 1 mM (cont.), 4 mM and 8 mM magnesium levels was greater than the inhibition in the root length of the seedlings grown in the same treatment solutions. The shoot length of the seedlings grown in 16 μM copper ion solution at 4 mM and 8 mM magnesium was 26 % ($p < 0.05$) and 71 % ($p < 0.01$) more than the shoot length of the seedlings grown in the same copper ion solution at 1 mM magnesium. Shoot lengths in the seedlings grown in all treatment solutions were greater at 4 mM magnesium than at 1 mM or 8 mM magnesium. However, the percentage inhibition in the shoot length of the seedlings grown in 8 μM, 16 μM and 32 μM copper solution relative to the shoot length in the seedlings grown in the control (1μM copper) was less at 8 mM magnesium.

In contrast to root length, the root fresh weight of the seedlings grown in the control (1 μM copper), 8 μ M and 16 μM copper solutions at 4 mM and 8 mM magnesium was greater than the root fresh weight of the seedlings grown in respective copper solutions at 1 mM (cont.) magnesium (Fig. 6.10). The percentage difference between root fresh weight of the seedlings grown in 8 μM and 16 μM copper was greater than the percentage difference between the root lengths of the seedlings grown at the same solutions. The percentage inhibition in root fresh weight of the seedlings grown at 8 μM, 16 μM and 32 μM copper relative to the respective control was less at 1 mM magnesium than at 4 mM or 8 mM magnesium. The magnesium-induced modification of copper ion toxicity in relation to shoot fresh weight of the seedlings followed a pattern similar to that which was observed for shoot length. The addition of magnesium at 4 mM and 8 mM relieved copper ion toxicity at 8 μM. The percentage inhibition in shoot fresh weight of the seedlings due to 8 μM copper relative to the respective control was less when the seedlings were grown at 8 mM magnesium than at 1 mM or 4 mM magnesium.

Figure 6.11 shows the effect of magnesium-induced modification of copper ion toxicity in relation to the root and shoot dry weight of the seedlings. The root dry weight of the seedlings grown in the control solution (1μM copper) at 4 mM and 8 mM magnesium was 35 % and 32 % more ($p < 0.05$) than the root dry weight of the seedlings grown in the control solution at 1 mM magnesium. The percentage inhibition of root dry weight of the seedlings grown in 8 μM copper solution relative

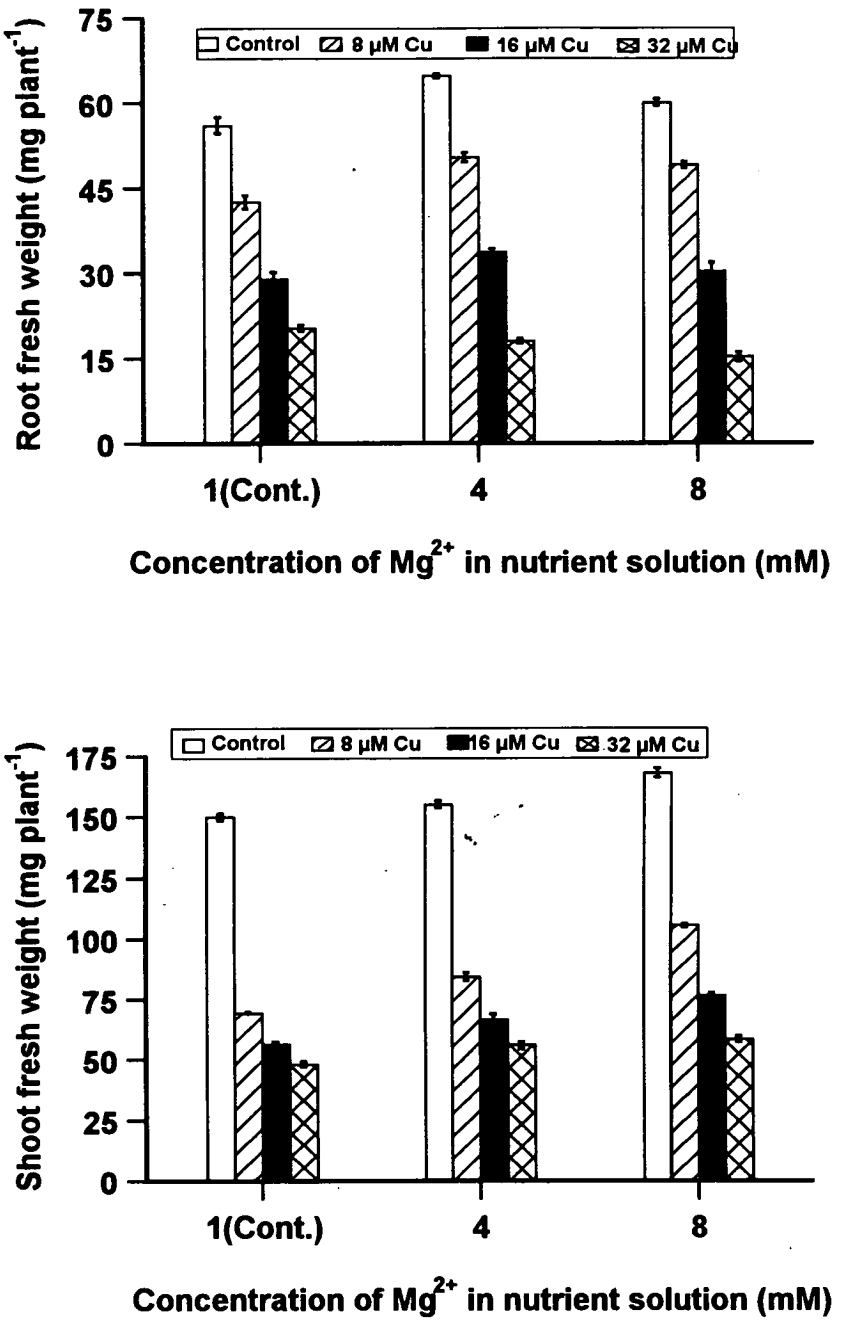


Fig. 6.10. Magnesium-induced modification of the effects of copper ion toxicity on root and shoot fresh weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

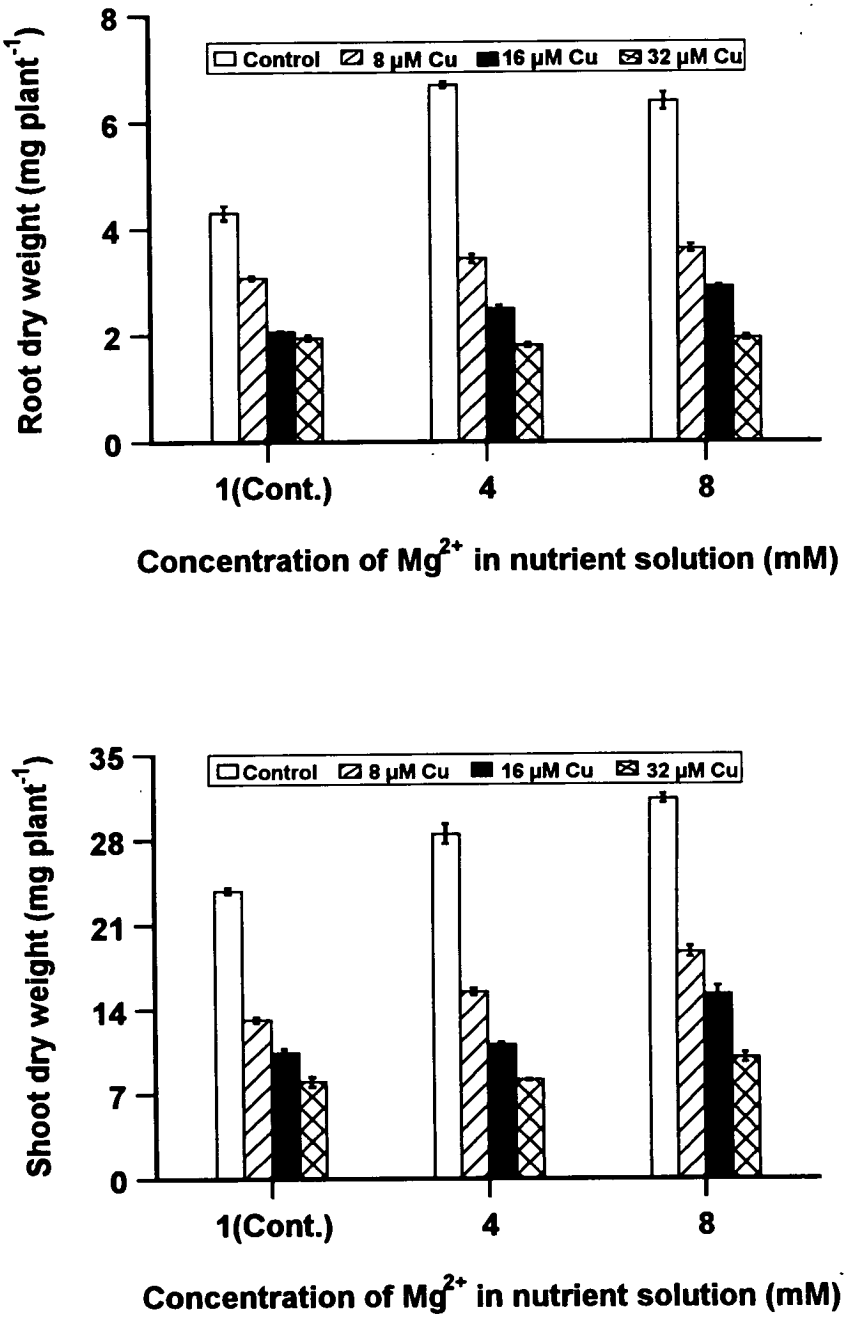


Fig. 6.11. Magnesium-induced modification of the effects of copper ion toxicity on root and shoot dry weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

to the respective control (1 μM copper) at 1 mM (cont.), 4 mM and 8 mM magnesium was greater than the percentage inhibition of root fresh weight of the seedlings grown in the same solutions. Maximum root dry weight of the seedlings was recorded when they were grown in the control solution (1 μM copper) at 4 mM magnesium, whereas, minimum root dry weight was observed when the seedlings were grown in 32 μM copper at 4 mM magnesium. Shoot dry weight of the seedlings grown in copper solutions at 1 mM (cont.), 4 mM and 8 mM magnesium followed a pattern similar to that which was observed for shoot length and shoot fresh weight of the seedlings grown in the same solutions. Shoot dry weight of the seedlings grown in the control solution (1 μM copper) at 4 mM and 8 mM magnesium was 20 % and 32 % greater ($p<0.05$) than the shoot dry weight of the seedlings grown in the control solution (1 μM copper) at 1 mM magnesium. Similarly, the shoot dry weight of the seedlings grown in 8 μM copper ions solution at 4 and 8 mM magnesium was 18 % and 42 % more than that of the seedlings grown in the same copper solutions at 1 mM (cont.) magnesium. The percentage inhibition in shoot dry weight of seedlings grown in 8 μM copper solution relative to the respective control (1 μM copper) was less at 1 mM magnesium (cont.) than at 4 mM or 8 mM magnesium.

The results obtained by using the computer simulation programme GEOCHEM-PC to investigate copper ion speciation at various magnesium and copper ion levels in the buffered nutrient solution are presented in Fig. 6.12. Detailed results in relation to all the nutrients are presented in Appendix V. When 1μM (cont.), 8 μM and 16 μM copper were present in addition to 1 mM (cont.), 4 mM and 8 mM magnesium, less than 0.25 % of the copper was present as the free metal ion. The remaining copper present was complexed with EDTA, and no precipitation of copper was predicted. When 32 μM copper was present in addition to magnesium at each of the 3 magnesium concentrations, it was found that less than 1 % copper was present as free metal ion, about 99 % was complexed with EDTA, and no precipitation of copper was predicted. Results obtained for magnesium speciation (Fig. 6.12) in the above treatment solutions showed that for each magnesium level more than 80 % of added magnesium was present as the free metal ion at all the copper ion levels.

The effect of magnesium ions on potassium leakage and on the amount of TBA-rm accumulated in the whole intact root systems of the seedlings is presented in

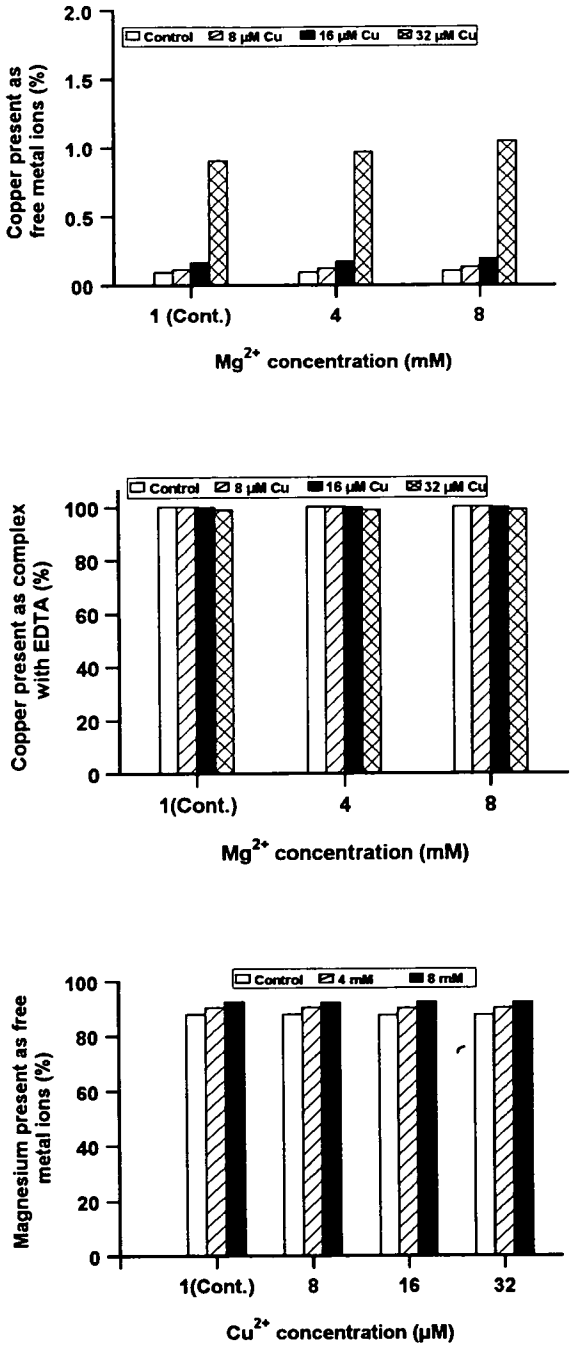
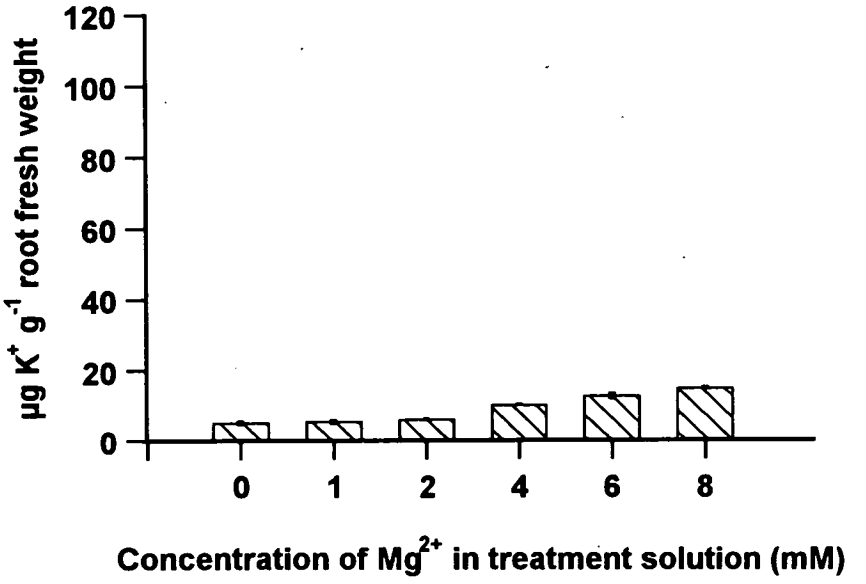


Fig. 6.12. Percentage of added copper and magnesium present in ionic form, and of copper complexed with EDTA in the complete Yoshida nutrient solution at different magnesium levels as predicted by GEOCHEM-PC.



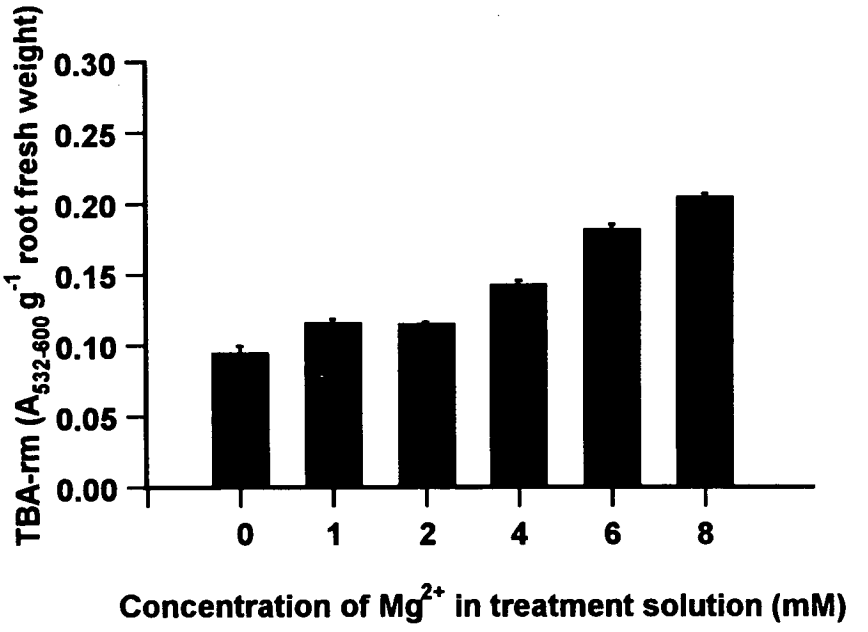


Fig. 6.13. Effect of different concentrations of magnesium ions on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

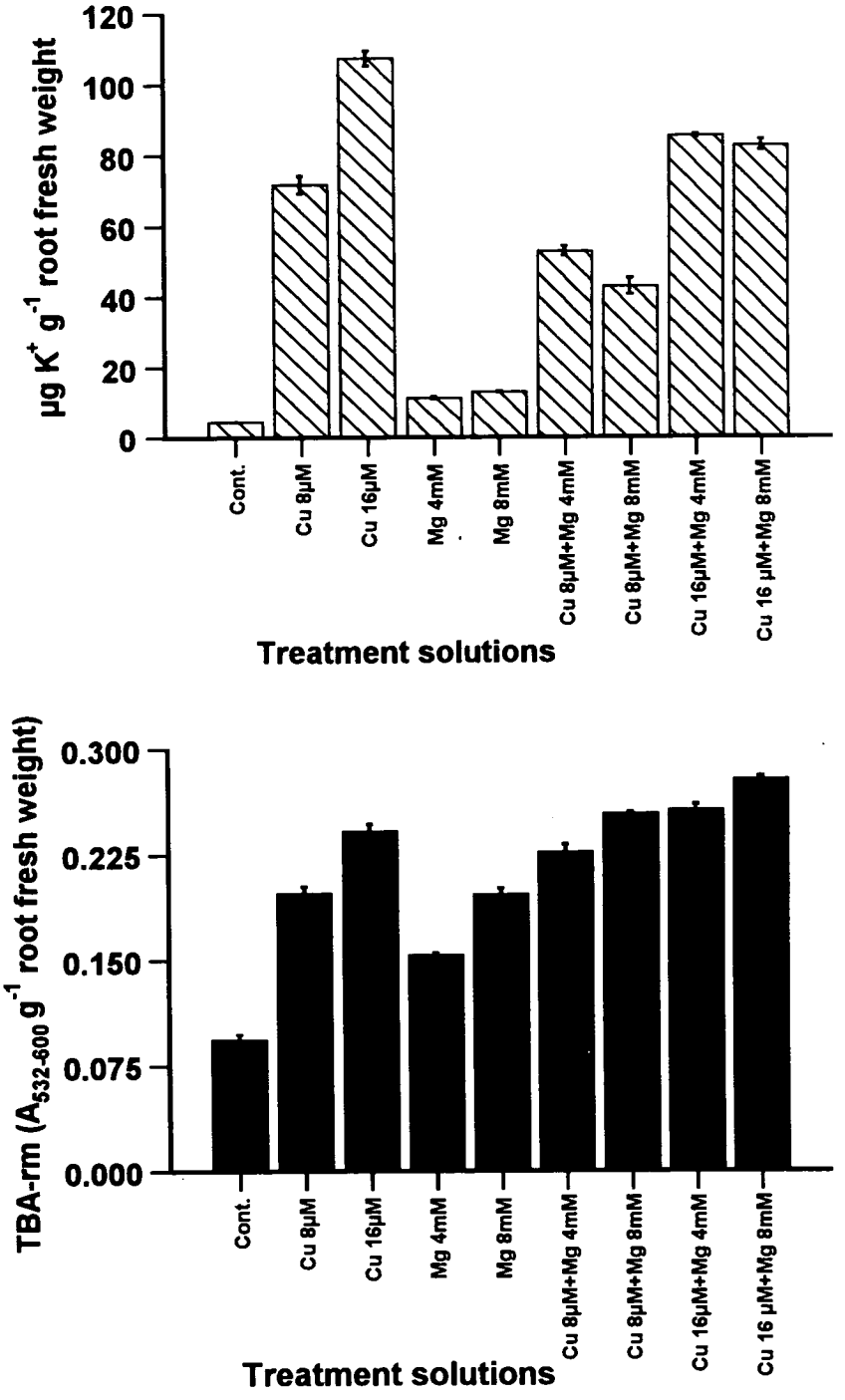


Fig. 6.14. Magnesium-induced modification of the effects of copper ion toxicity on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

Fig. 6.13. The amount of potassium ions detected in 4 and 8 mM magnesium solutions after incubation of the whole intact root systems of the seedlings was 90 % and 185 % more ($p<0.01$) than that measured in the 1 mM magnesium . Similarly, TBA-rm accumulated in the roots at 4 and 8 mM magnesium was 25 % and 75 % more ($p<0.01$) than the TBA-rm accumulated in the root at 1 mM magnesium solution.

The magnesium-induced modification in the amount of potassium ion leakage and TBA-rm accumulation due to copper ion toxicity in the whole intact root systems of seedlings is presented in Fig. 6.14. The amount of potassium ions leaked from seedlings incubated in the 8 μM copper + 4 mM magnesium and 8 μM copper + 8 mM magnesium solutions was 26 % and 40 % less ($p<0.05$) than the amount of potassium ions leaked from seedlings incubated in the 8 μM copper solution. The amount of potassium ions leaked from seedlings incubated in the 16 μM copper + 4 mM magnesium and 16 μM copper + 8 mM magnesium solutions was 20 % and 23 % less ($p<0.05$) than the amount of potassium ions leaked from seedlings incubated in the 16 μM copper ion solution.

The amount of TBA-rm in the roots after incubation with 8 μM copper + 4 mM magnesium and with 8 μM copper + 8 mM magnesium was 15 % and 25 % more ($p<0.05$) than the amount of TBA-rm in the roots after they had been incubated in 8 μM copper. When the roots were incubated in the 16 μM copper + 4 mM magnesium solutions, the amount of TBA-rm was only slightly higher than that of the roots after they had been incubated in 16 μM copper, however, when the roots of the seedlings were incubated in the 16 μM copper + 8 mM magnesium solution TBA-rm was 15 % more ($p<0.05$) than that in the roots of the seedlings incubated in the 16 μM copper.

6.4. Citrate-induced modification of copper ion toxicity in seedlings of rice *(Oryza sativa* L.) cv. NIAB 6.

6.4.1. Experimental Procedure

A preliminary experiment was carried out to investigate the effect of citrate on root elongation of rice seedlings. The procedure described in Section 2.2.1 was followed for the growth of the seedlings, except that the sheets of seeds were placed in the specimen tubes containing buffered Yoshida nutrient solution pH 5.5, with 0, 1,

5, 10, 20, 30, 40 and 50 μM citrate. After 15 d the seedlings were removed from the specimen tubes, the length of the longest root was measured, and a dose-response curve was plotted (Fig. 6.1.).

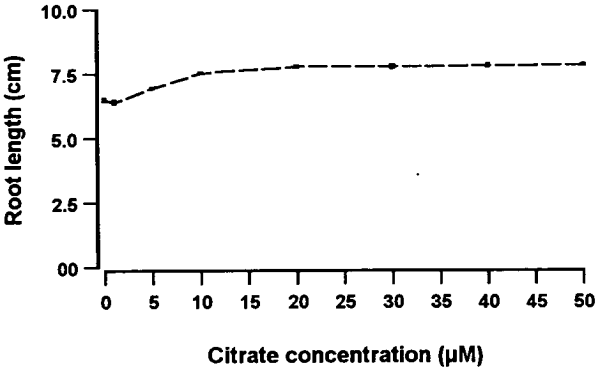


Fig. 6.15. Dose-response curve of the mean length of the longest root of 15 d seedlings of rice (*Oryza sativa* L.) grown in Yoshida nutrient solution supplemented with different concentrations of citrate.

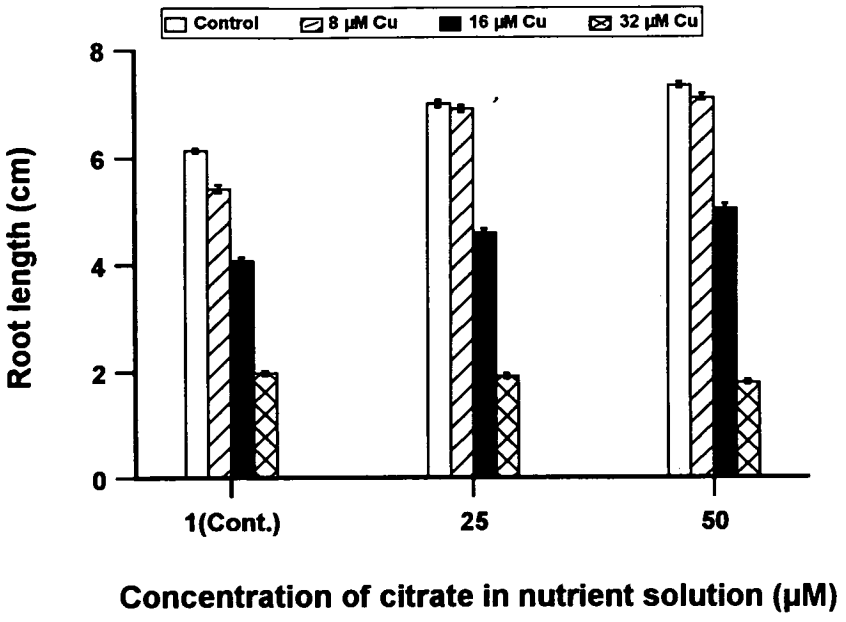
In the next experiment the seedlings were grown in buffered Yoshida nutrient solution pH 5.5, containing, 1 (control), 8, 16 and 32 μM $CuSO_4$ at each 1 (control), 25 and 50 μM citrate. Growth conditions and the experimental procedures were the same as those described in Section 2.2.1. After 15 d, the length of the shoot and of the longest root and the total root and shoot fresh and dry weights were measured following the method described in Section 2.2.1.

In order to determine the effect of citrate on copper ion-induced potassium leakage and on lipid peroxidation in roots, seedlings were grown for 15 d in the control nutrient solution. The whole root systems of intact plants were pre-incubated for potassium loading as described in Section 2.7. The seedlings were then divided into two sets. The root systems of set one were transferred to 100 ml of 0 (ultra-pure water), 1, 5, 10, 20, 30, 40 and 50 μM citrate and, the root systems of set two were transferred to 100 ml of 0 (ultra-pure water, used as control), 25 and 50 μM citrate and 8 and 16 μM $CuSO_4$ alone, and in combinations, for 16 h. The concentration of potassium ions in the solution at the end of 16 h period was measured by the procedure described in Section 2.7, and the TBA-rm content of the same roots was measured following the method described in Section 2.8.

6.4.2. Results

The citrate-induced modification of the effects of copper ion toxicity on the length of the longest root and of the shoot of rice seedlings is presented in Fig. 6.16. The effect of copper solutions on the root length of the seedlings grown in Yoshida nutrient solution containing 1 μM citrate (control) was similar to that which was reported in Section 4.4.2 (Fig 4.10) for seedlings grown in complete Yoshida solution without citrate. The citrate copper interaction was non-significant. The root lengths of the seedlings grown in the control (1μM copper), 8 μM and 16 μM copper solution at 50 μM citrate was greater than the root lengths of the seedlings grown in the same copper solutions at 1 μM citrate (cont.) or 25 μM citrate. When the seedlings were grown in 32 μM copper ion solution the root length was approximately the same in all concentrations of citrate. The percentage inhibition of root elongation in the seedlings grown in 16 μM copper solution relative to the root elongation of the seedlings in the respective control (1μM copper) solution was less at 50 μM citrate than at 25 μM citrate. The effect of copper solutions on the shoot length of the seedlings grown in 1 μM (cont.), 25 μM and 50 μM citrate followed a pattern which was similar to that observed for the root length. The percentage difference between the shoot length of the seedlings grown in 8 μM and 16 μM copper solution was less than the percentage difference between the root lengths of the seedlings grown at the same copper solutions. Shoot lengths in the seedlings grown in control (1 μM copper), 8 μM and 16 μM copper were greater at 50 μM citrate than at 1 μM citrate (cont.) or 25 μM citrate. The percentage inhibition in the shoot length of the seedlings grown in 8 μM and 16 μM copper solution relative to the shoot length of the seedlings grown in respective control (1 μM copper) solution was less at 50 μM citrate than at 25 μM citrate.

The percentage difference in root fresh weights of the seedlings grown in the control (1 μM copper) solution at 25 μM and 50 μM citrate relative to the root fresh weight of the seedlings grown in 1 μM citrate was less than the percentage difference in root length of the seedlings grown in same solutions (Fig. 6.17). When the seedlings were grown in 16 μM copper ion solution at 25 μM and 50 μM citrate the root fresh weight of the seedlings were similar to the root fresh weight of the seedlings grown in the same copper ion solution at 1 μM citrate. The percentage inhibition in root fresh weight of the seedlings grown in 8 μM and 16 μM copper



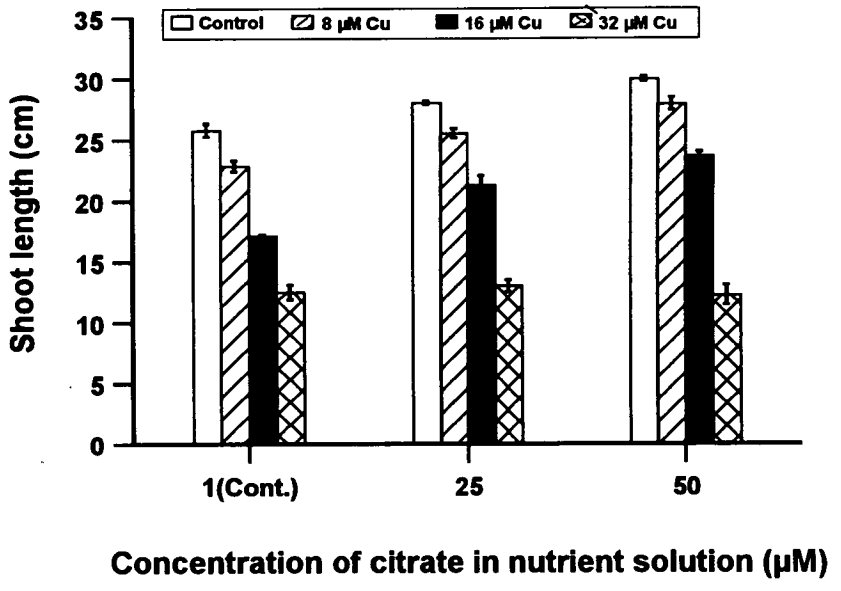


Fig. 6.16. Citrate-induced modification of the effects of copper ion toxicity on the length of the longest root and of the shoot of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

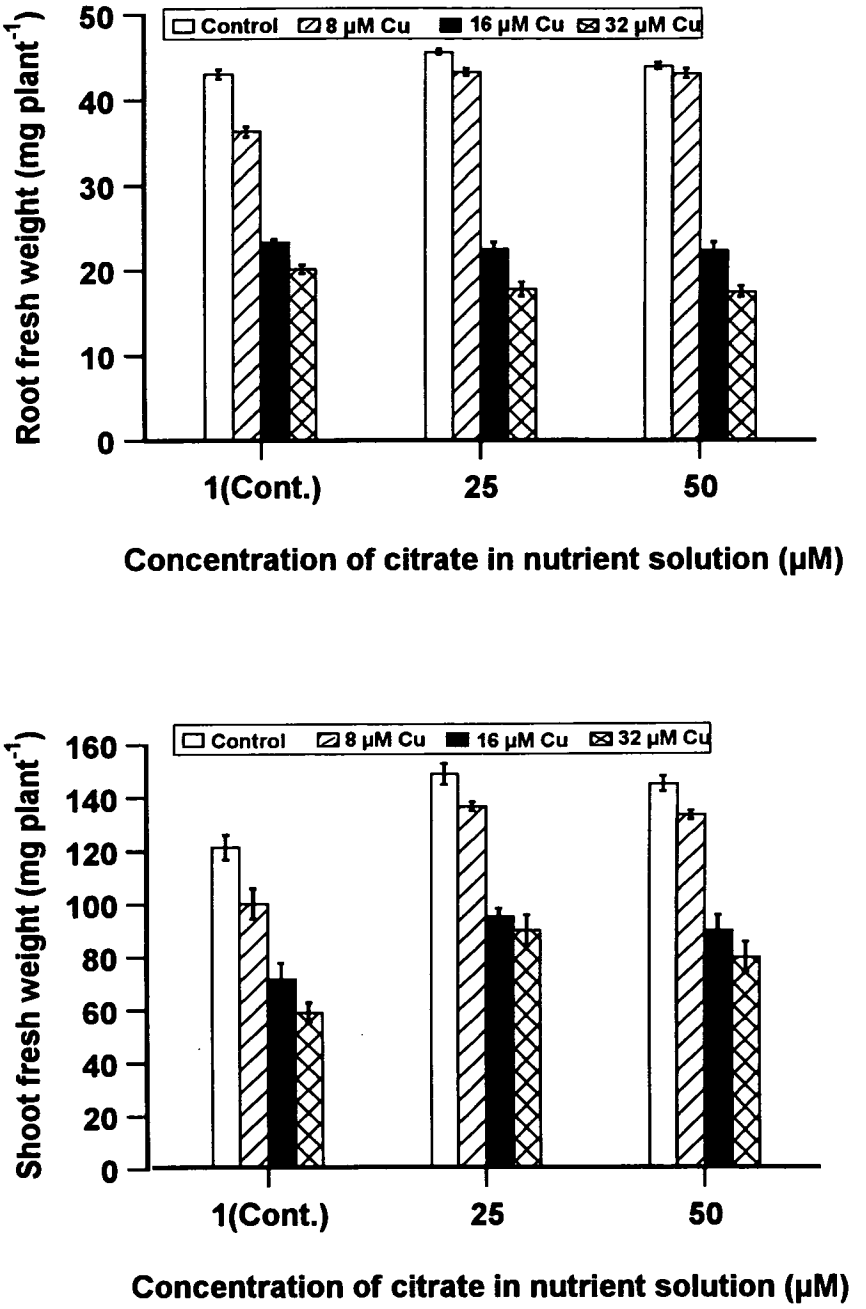


Fig. 6.17. Citrate-induced modification of the effects of copper ion toxicity on root and shoot fresh weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

solution relative to the respective controls was less at 50 μM citrate than at 25 μM citrate. The percentage difference between shoot fresh weight of the seedlings grown in 8 μM and 16 μM copper solution at 25 μM and 50 μM citrate was greater than the percentage difference between the shoot lengths of the seedlings grown in the same solutions. The percentage inhibition in the shoot fresh weight of the seedlings due to 8 μM and 16 μM copper relative to respective control was less when the seedlings were grown at 25 μM citrate than at 50 μM citrate.

Figure 6.18 shows the effect of citrate ions on copper ion-induced toxicity in relation to the root dry weight of the seedlings. The root dry weights of the seedlings grown in the control solution (1μM copper) and 8 μM copper solution at 25 μM and 50 μM citrate followed a pattern similar to that which was observed for root fresh weight when the seedlings were grown in the same solutions. However, the percentage difference in root dry weight of the seedlings grown in 8 μM and 16 μM copper solution at 25 μM and 50 μM citrate was less than the percentage difference between the root fresh weight of the seedlings at the same treatments. When the seedlings were grown in 32 μM copper ion solution at 25 μM citrate the root dry weight of the seedlings was 32 % more ($p < 0.05$) than that of the seedlings grown in the same copper ion solution at 1 μM citrate. The percentage inhibition in the root dry weight of the seedlings grown in 8 μM and 16 μM copper solution relative to respective control was less at 50 μM citrate than at 25 μM citrate. Shoot dry weights of the seedlings grown in copper solutions at 1 μ M (cont.), 25 μM and 50 μM citrate followed a pattern similar to that which was observed for root fresh weight. Shoot dry weight of the seedlings grown in the control solution (1 μM copper) at 25 μM and 50 μM citrate was 15 % and 13 % more ($p<0.05$) than the shoot dry weight of the seedlings grown in the control solution (1 μM copper) at 1 μM citrate. When the seedlings were grown in 16 μM copper ion solution at 25 μM and 50 μM citrate the shoot dry weights were 33 % and 43 % more ($p < 0.05$) than that of the seedlings grown in the respective treatment at 1 μM citrate. The percentage inhibition in the shoot dry weight of the seedlings grown in 8 μM and 16 μM copper solution relative to the respective control (1 μM copper) was less at 50 μM citrate than at 25 μM citrate.

The results obtained by using the computer simulation programme GEOCHEM-PC to investigate copper ion speciation at various citrate and copper ion

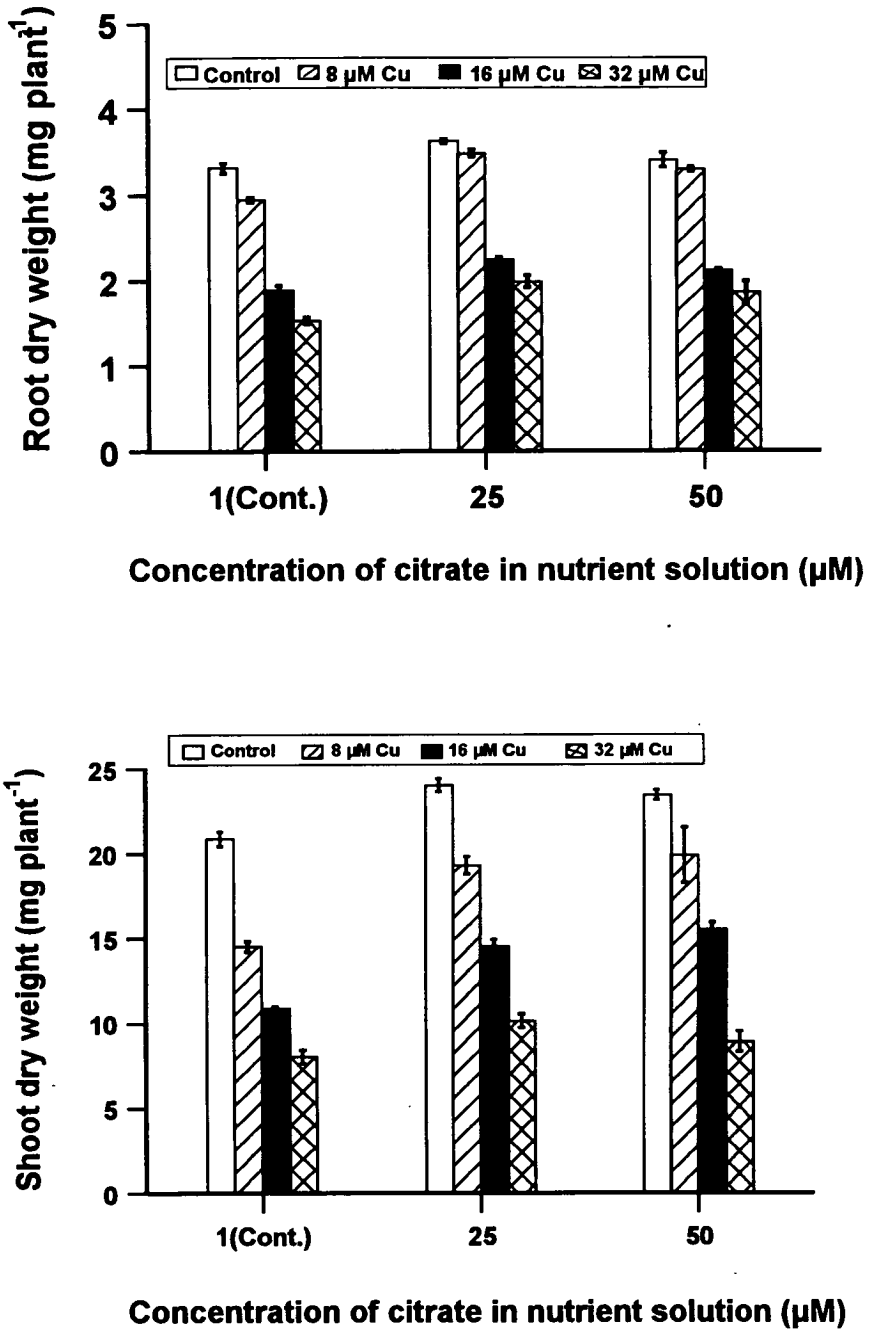


Fig. 6.18. Citrate-induced modification of the effects of copper ion toxicity on root and shoot dry weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

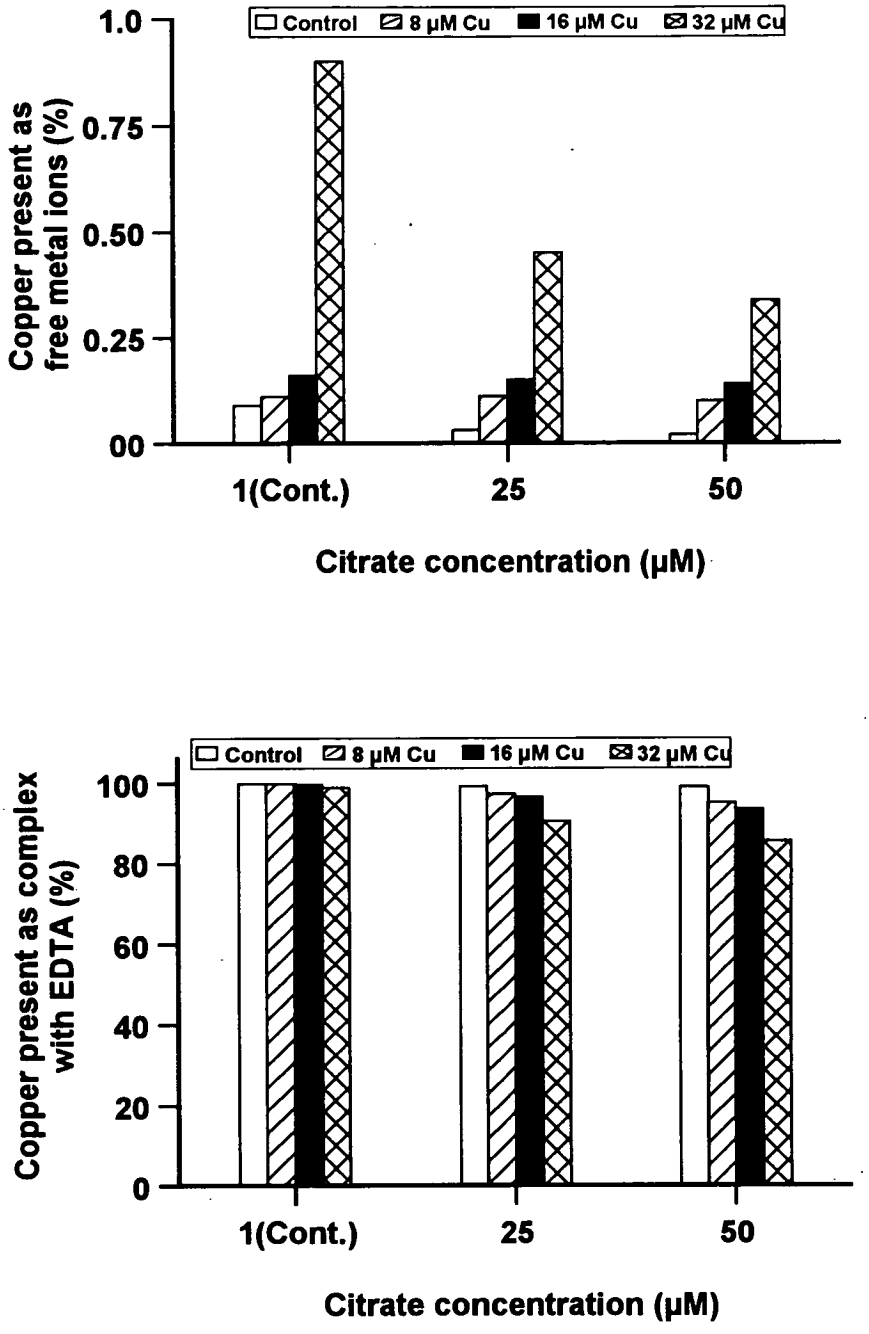


Fig. 6.19. Percentage of added copper present in ionic form and complexed with EDTA in complete Yoshida nutrient solution at different citrate levels as predicted by GEOCHEM-PC.

levels in the buffered nutrient solution are presented in Fig. 6.19. Detailed results in relation to all the nutrients are presented in Appendix VI. When 1 (cont.), 8 μM and 16 μM copper were present in addition to 1 μM (cont.), 25 μM and 50 μM citrate, less than 0.25 % copper was present as free metal ion. The remaining copper present was complexed with EDTA, and no precipitation of copper was predicted. When 32 μM copper was present in addition to citrate at each of the 3 citrate concentrations, it was found that less than 1 % copper was present as free metal ion, about 99 % copper was complexed with EDTA, and no precipitation of copper was predicted.

The effect of citrate ions on potassium leakage and on the amount of TBA-rm accumulated in the whole intact root systems of the seedlings is presented in Fig. 6.20. The amount of potassium ions detected in the 20 μM and 50 μM citrate solutions after incubation of the whole intact root systems of the seedlings was 163 % and 171 % more ($p<0.01$) than that measured in the 1 μM citrate. Similarly, TBA-rm accumulated in the roots at 20 μM and 50 μM citrate solution was 27 % and 76 % more ($p<0.05$) than the TBA-rm accumulated in the root at 1 μM citrate.

The citrate-induced modification in the amount of potassium ion leakage and TBA-rm accumulation due to copper ion toxicity in the whole intact root systems of seedlings is presented in Fig. 6.21. The amount of potassium ions leaked from seedlings incubated in the 8 μM copper + 25 μM citrate and 8 μM copper + 50 μM citrate solutions was 43 % and 41 % less ($p<0.05$) than the amount of potassium ions leaked from seedlings incubated in the 8 μM copper solution. The amount of potassium ions leaked from seedlings incubated in the 16 μM copper + 25 μM citrate and 16 μM copper + 50 μM citrate solutions was 38 % and 52 % less ($p<0.05$) than the amount of potassium ions leaked from seedlings incubated in 16 μM copper ion solution. However, the amount of potassium ions leaked into the 16 μM copper + 50 μM citrate solution was 22 % less ($p < 0.05$) than the amount of potassium ions leaked into the 16 μM copper + 25 μM citrate solution.

The amount of TBA-rm in the roots after incubation with 8 μM copper + 25 μM citrate was less than that of 8 μM copper alone, and 8 μM copper + 50 μM citrate was similar to the amount of TBA-rm in the roots after they had been incubated in the 8 μM copper. When the roots were incubated in the 16 μM copper + 25 μM citrate and 16 μM copper + 50 μM citrate solutions, the amount of TBA-rm

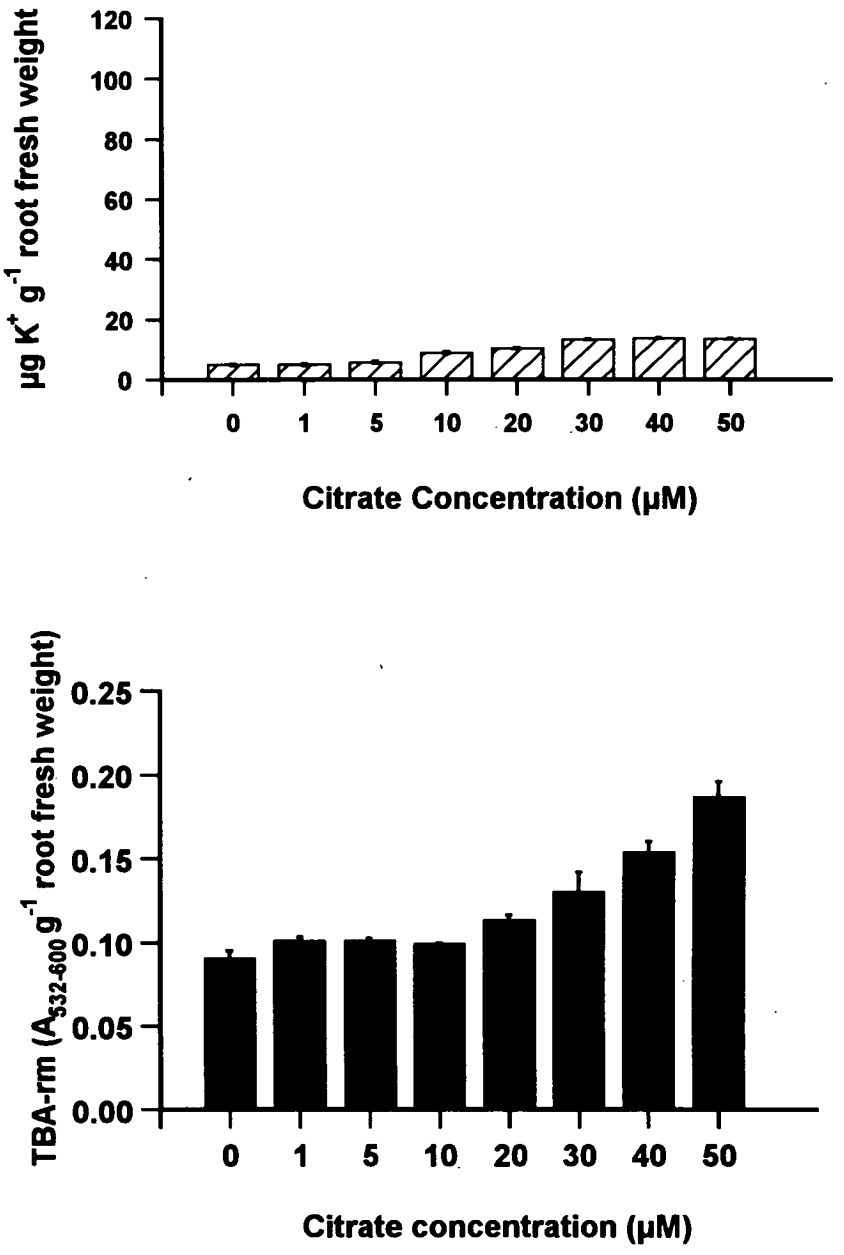


Fig.6.20. Effect of different concentrations of citrate on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

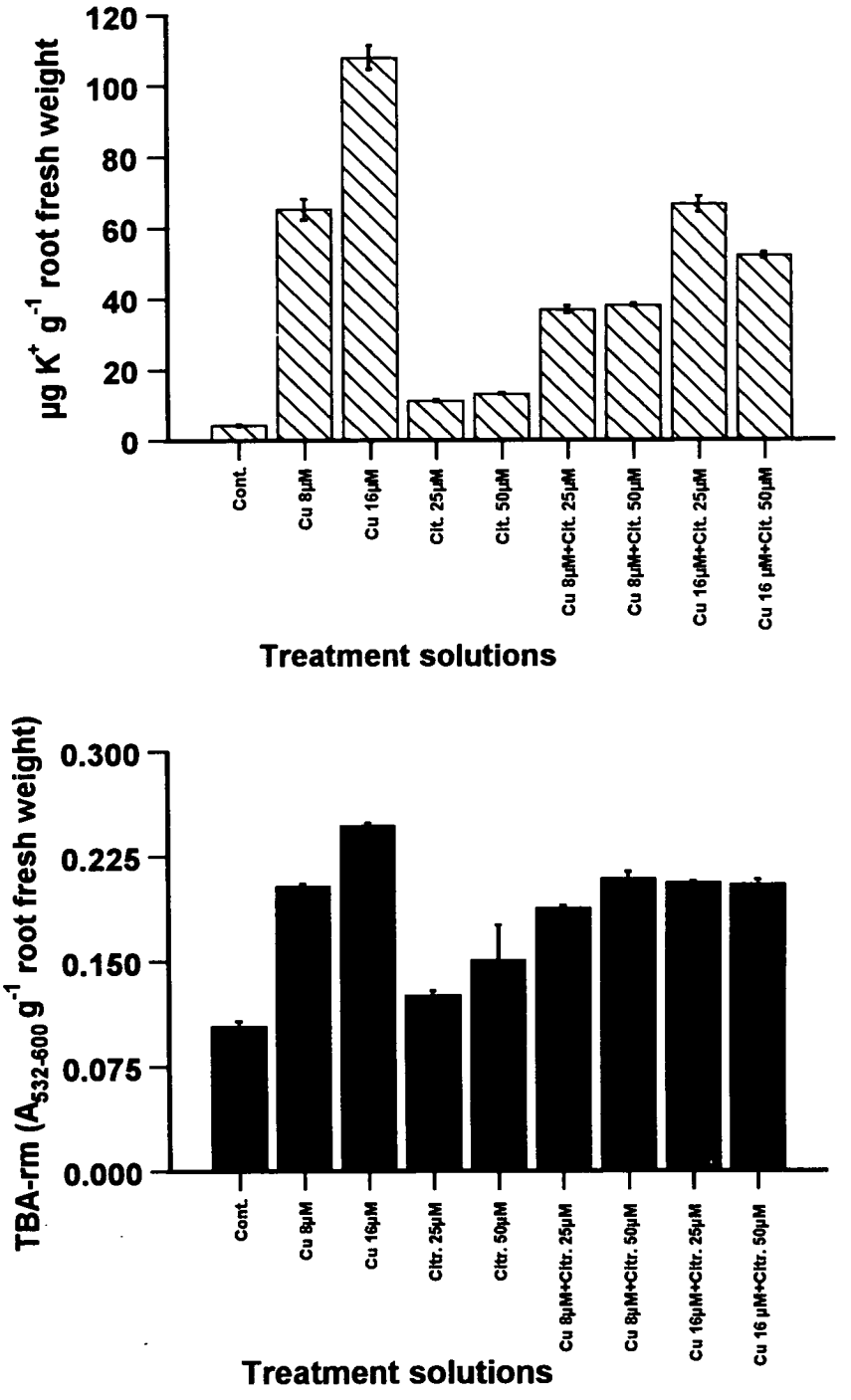


Fig. 6.21. Citrate-induced modification of the effects of copper ion toxicity on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

was 17 % less ($p < 0.05$) than that of the roots after they had been incubated in the 16 μM copper. The amount of TBA-rm in the roots of the seedlings incubated in the 8 μM copper + 50 μM citrate solution was 11 % more ($p < 0.05$) than that in the roots when they were incubated in the 8 μM copper + 25 μM citrate solution. When the roots were incubated in the 16 μM copper + 50 μM citrate solution the amount of TBA-rm was similar to that in the root when they were incubated in the 16 μM copper + 25 μM citrate solution.

6.5. Oxalate-induced modification of copper ion toxicity in seedlings of rice *(Oryza sativa* L.*)* cv. NIAB 6.

6.5.1. Experimental Procedure

A preliminary experiment was carried out to investigate the effect of oxalate on root elongation of rice seedlings. The procedure described in Section 2.2.1 was followed for the growth of the seedlings, except that the sheets of seeds were placed in specimen tubes containing buffered Yoshida nutrient solution pH 5.5, with 0, 1, 5, 10, 20, 30, 40 and 50 μM oxalate. After 15 d, the seedlings were removed from the specimen tubes and the length of the longest root was measured and a dose-response curve was plotted (Fig. 6.22).

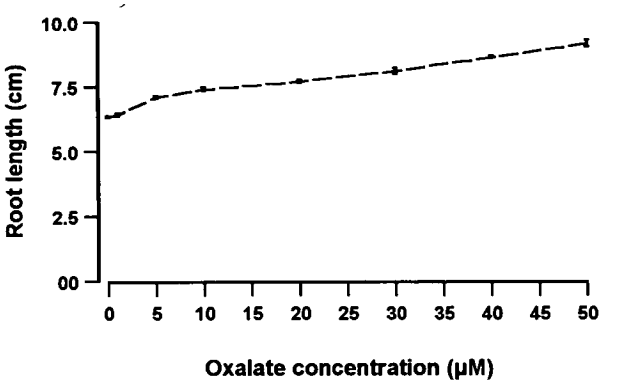


Fig. 6.22. Dose-response curve of the mean length of the longest root of 15 d seedlings of rice (*Oryza sativa* L.) grown in Yoshida nutrient solution supplemented with different concentrations of oxalate.

In the next experiment the seedlings were grown in buffered Yoshida nutrient solution pH 5.5, containing, 1 (control), 8, 16 and 32 μM $CuSO_4$, each at 1 (control), 25 and 50 μM oxalate. Growth conditions and the experimental procedures were the same as those described in Section 2.2.1. After 15 d, the length of the shoot and of the longest root and the total root and shoot fresh and dry weight were measured following the methods described in Section 2.2.1.

In order to determine the effect of oxalate on copper ion-induced potassium leakage and on lipid peroxidation in roots, seedlings were grown for 15 d in the control nutrient solution. The whole root systems of intact plants were pre-incubated for potassium loading as described in Section 2.7. The seedlings were then divided into two sets. The root systems of set one were transferred to 100 ml of the 0 (ultra-pure water), 1, 5, 10, 20, 30, 40 and 50 μM oxalate and the root systems of set two were transferred to 100 ml 0 (ultra-pure water, used as control), of 25 and 50 μM oxalate, and 8 and 16 μM $CuSO_4$ alone, and in combinations, for 16 h. At the end of 16 h period the concentration of potassium ions in the solution was measured by the procedure described in Section 2.7, and the TBA-rm content of the same roots was measured following the method described in Section 2.8.

6.5.2. Results

The oxalate-induced modification of the copper ion toxicity in relation to the length of the longest root and of the shoot of rice seedlings is presented in Fig. 6.23. Interaction between copper and oxalate was non-significant. The root length of the seedlings grown in 8 μM copper solution at 25 μM and 50 μM oxalate were 30 % and 32 % more ($p<0.05$) than the root length of the seedlings grown in the same copper solution at 1 μM oxalate. When the seedlings were grown in 16 μM copper solution at 25 μM and 50 μM oxalate the root length was 22 % and 30 % greater ($p < 0.05$) than the root length of the seedlings grown in the same copper solution at 1 μM oxalate. When the seedlings were grown in 32 μM copper solution the root length of the seedlings was approximately the same in all concentrations of oxalate. The percentage inhibition in the root length of the seedlings grown in 16 μM copper solution relative to the root length of the seedlings in the respective control (1 μM copper) solution was less at 50 μM oxalate than at 25 μM oxalate. The effect of oxalate on copper-induced toxicity in relation to shoot length of the seedlings followed a pattern similar to that which was observed for root

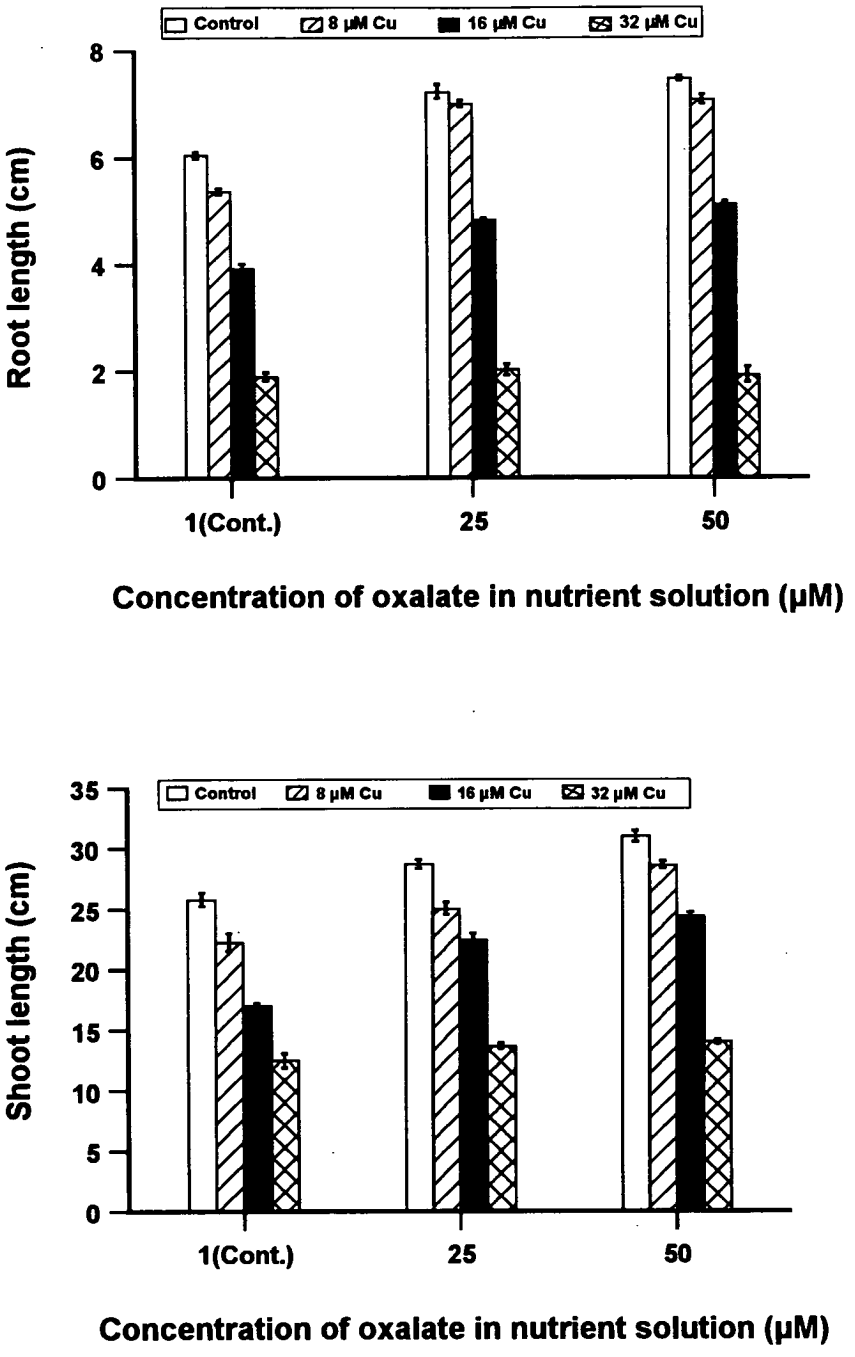


Fig. 6.23. Oxalate-induced modification of the effect of copper ion toxicity on the length of the longest root and of the shoot of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

length. The percentage difference between the shoot length of the seedlings grown in 8 μM and 16 μM copper solution at 25 μM and 50 μM oxalate was smaller than the percentage difference between the root elongation of the seedlings grown in the same solutions. The shoot lengths of the seedlings grown in copper solutions at 50 μM oxalate were longer than the shoot lengths of the seedlings grown in the same copper solutions at 1 μM (cont.) and 25 μM oxalate. The percentage inhibition in the shoot length of the seedlings grown in 8 μM and 16 μM copper solution relative to the respective control was smaller at 50 μM oxalate than at 25 μM oxalate.

The root fresh weight of the seedlings grown in copper solutions at 1 μM oxalate (cont.), 25 μM and 50 μM oxalate is presented in Fig 6.24. The percentage inhibition in root fresh weights of the seedlings grown in 16 μM copper solution at 25 μM and 50 μM oxalate was greater than the percentage inhibition in root length of the seedlings grown in the same oxalate solutions. Similarly the percentage difference between root fresh weight of the seedlings grown in 16 μM and 32 μM copper at all the oxalate solutions was smaller than the percentage differences between the root lengths of the seedlings grown in the control treatments solutions. The percentage difference in shoot fresh weight of the seedlings grown in 8 μM and 16 μM copper solution at 25 μM and 50 μM oxalate was greater than the percentage difference between the shoot length of the seedlings grown in the same oxalate concentration at control solutions. Whereas, the percentage difference between the shoot fresh weight of the seedlings grown in 16 μM and 32 μM copper solution at 25 μM and 50 μM oxalate was less than the percentage difference between the shoot lengths of the seedlings grown in the same solution. The percentage inhibition in the shoot elongation of the seedlings grown in 8 μM and 16 μM copper solution relative to the respective control was less when seedlings were grown at 25 μM than at 50 μM oxalate.

Figure 6.25 shows the effect of oxalate ions on copper-induced toxicity in relation to the root and shoot dry weight of the seedlings. The root dry weight of the seedlings grown in copper solutions at 1 μM oxalate (cont.) solution, 25 μM and 50 μM oxalate followed a pattern very similar to that which was observed for the root fresh weight of the seedlings grown in the same solutions. The percentage inhibition in the root dry weight of the seedlings grown in 8 μM and 16 μM copper solution relative to the respective control was less at 50 μM oxalate than at 25 μM oxalate.

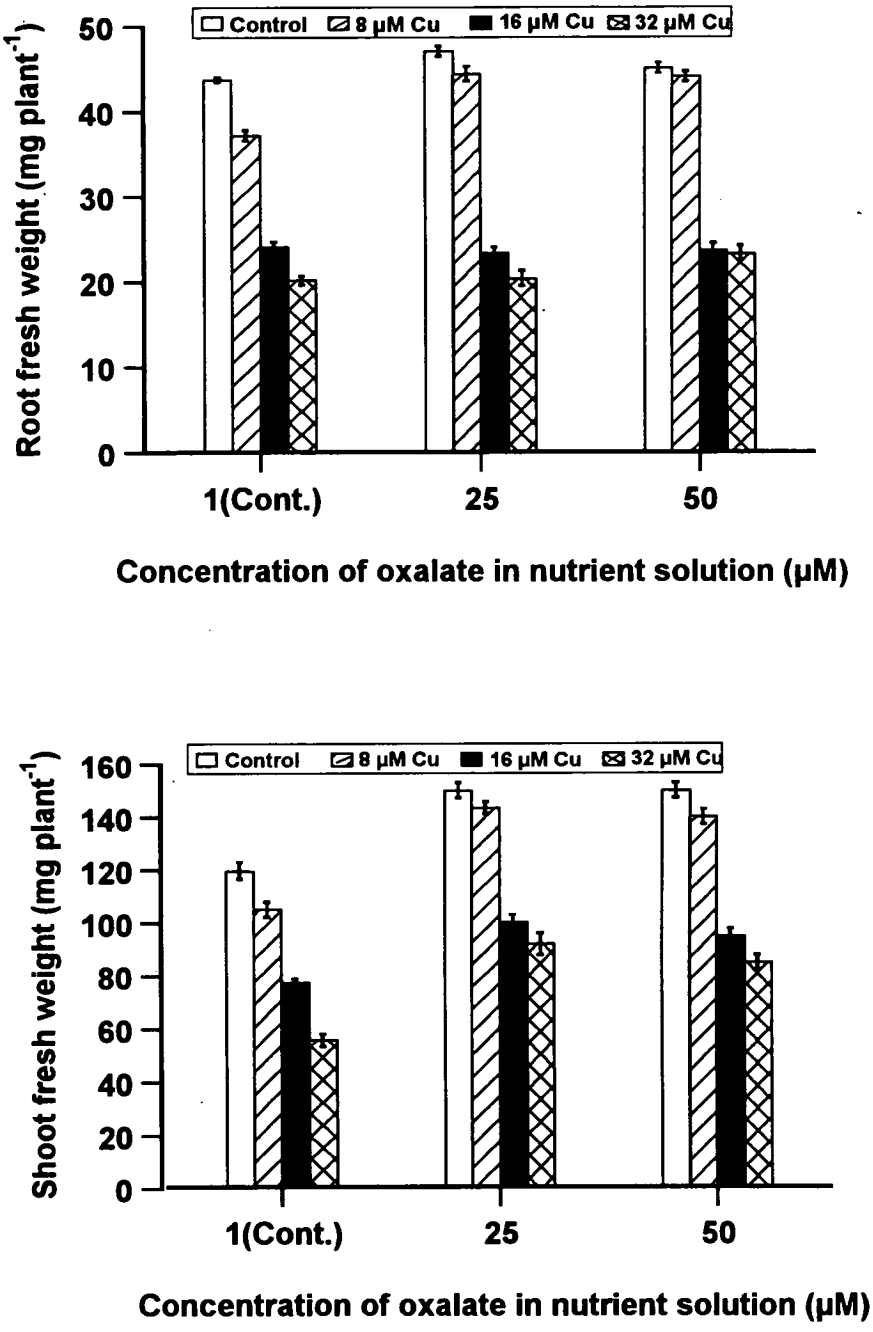


Fig. 6.24. Oxalate-induced modification of the effects of copper ion toxicity on root and shoot fresh weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

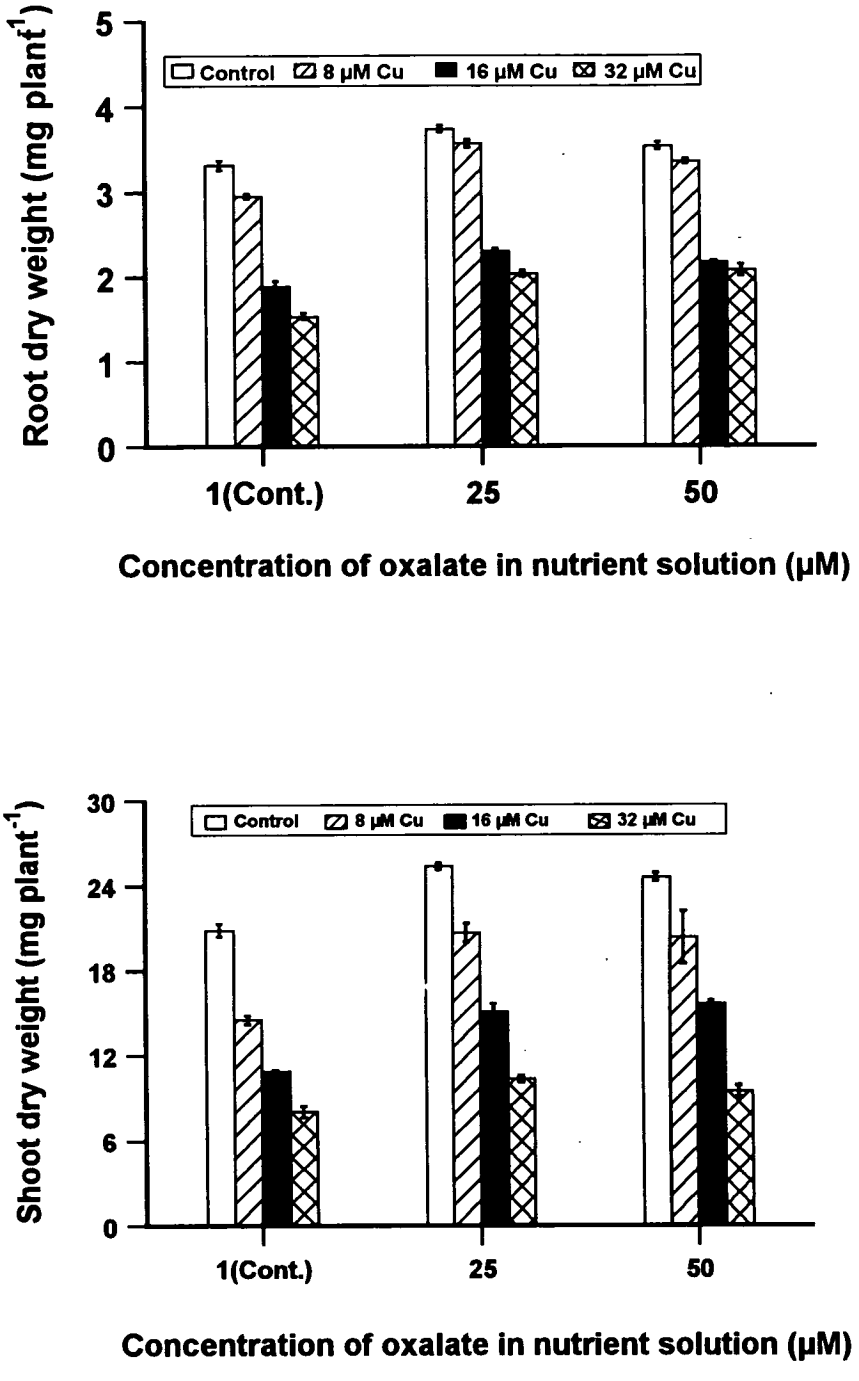


Fig. 6.25. Oxalate-induced modification of the effect of copper ion toxicity on root and shoot dry weight of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

The percentage difference between the shoot dry weight of the seedlings grown in 16 μM copper and 32 μM copper solution at 25 μM and 50 μM oxalate was greater than the percentage difference between the shoot fresh weight of the seedlings grown in the same copper solution at 1 μM oxalate. The percentage inhibition in shoot dry weight of the seedlings grown in 8 μM copper and 16 μM copper solution relative to the respective control (1μM copper) was less when the seedlings were grown at 50 μM oxalate than at 25 μM oxalate.

The results obtained by using the computer simulation programme GEOCHEM-PC to investigate copper ion speciation at various oxalate and copper ion levels in the buffered nutrient solution are presented in Fig. 6.26. Detailed results in relation to all the nutrients are presented in Appendix VI. When 1 (cont.), 8 μM and 16 μM copper were present in addition to 1 μM (cont.), 25 μM and 50 μM oxalate, less than 0.25 % copper was present as free metal ion. The remaining copper present was complexed with EDTA, and no precipitation of copper was predicted. When 32 μM copper was present in addition to oxalate at each of the 3 oxalate concentrations, it was found that less than 1 % copper was present as free metal ion, about 99 % of the copper was complexed with EDTA, and no precipitation of copper was predicted.

The effect of oxalate ions on potassium leakage and on the amount of TBA-rm accumulated in the whole intact root systems of the seedlings is presented in Fig. 6.27. The amount of potassium ions detected in 20 μM and 50 μM oxalate solutions after incubation of the whole intact root systems of the seedlings was 122 % and 156 % more ($p<0.01$) than that measured in the 1 μM oxalate. Similarly, TBA-rm accumulated in the roots at 20 μM and 50 μM oxalate was 21 % ($p < 0.05$) and 108 % more ($p<0.01$) than the TBA-rm accumulated in the root at 1 μM oxalate.

The oxalate-induced modification in the amount of potassium ion leakage and TBA-rm accumulation due to copper ion toxicity in the whole intact root systems of seedlings is presented in Fig. 6.28. The amount of potassium ions leaked from seedlings incubated in the 8 μM copper + 25 μM oxalate and 8 μM copper + 50 μM oxalate solutions was 23 % and 24 % less ($p<0.05$) than the amount of potassium ions leaked from seedlings incubated in the 8 μM copper solution. The amount of potassium ions leaked from seedlings incubated in the 16 μM copper + 25 μM oxalate

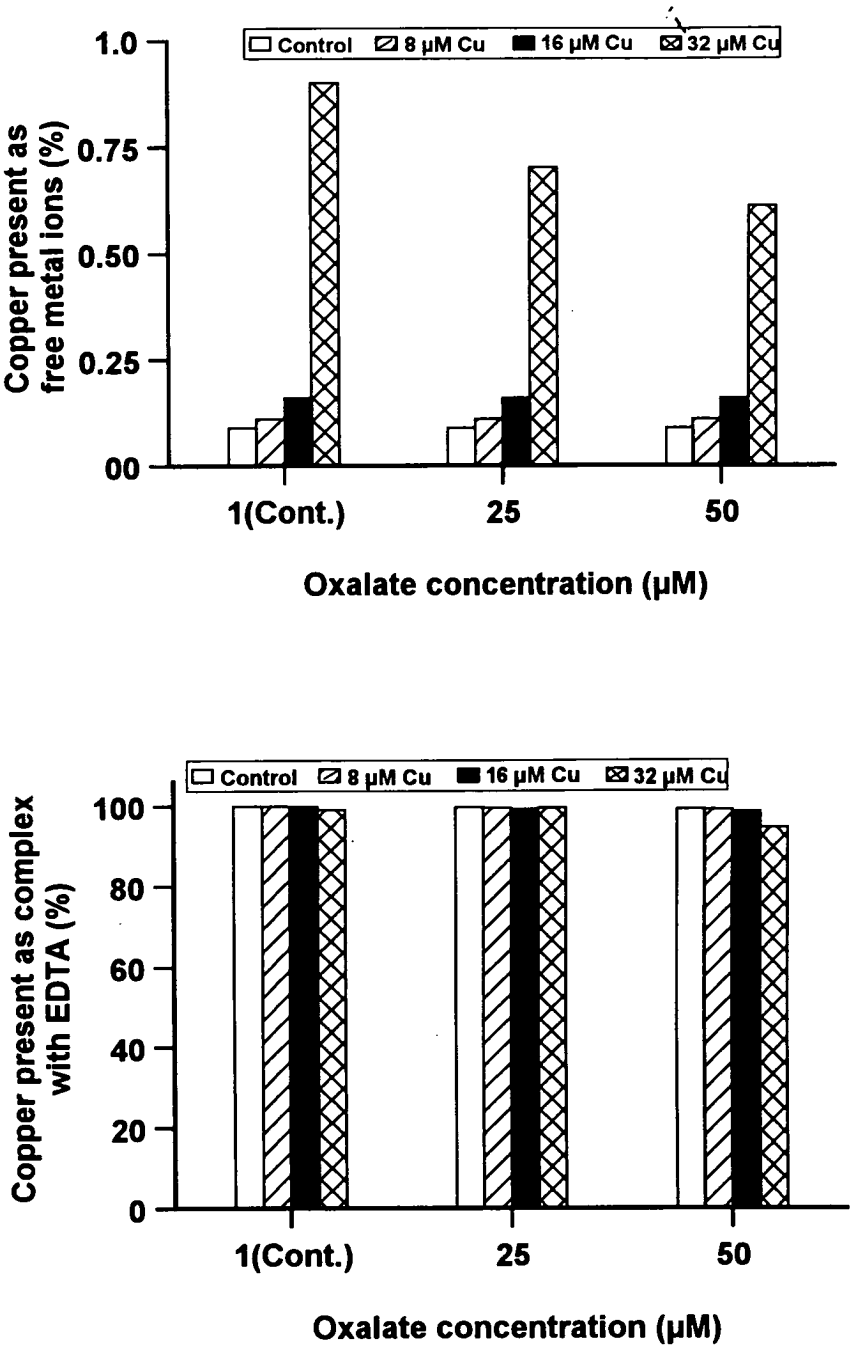


Fig. 6.26. Percentage of added copper present in ionic form and complexed with EDTA in complete Yoshida nutrient solution at different oxalate levels as predicted by GEOCHEM-PC.

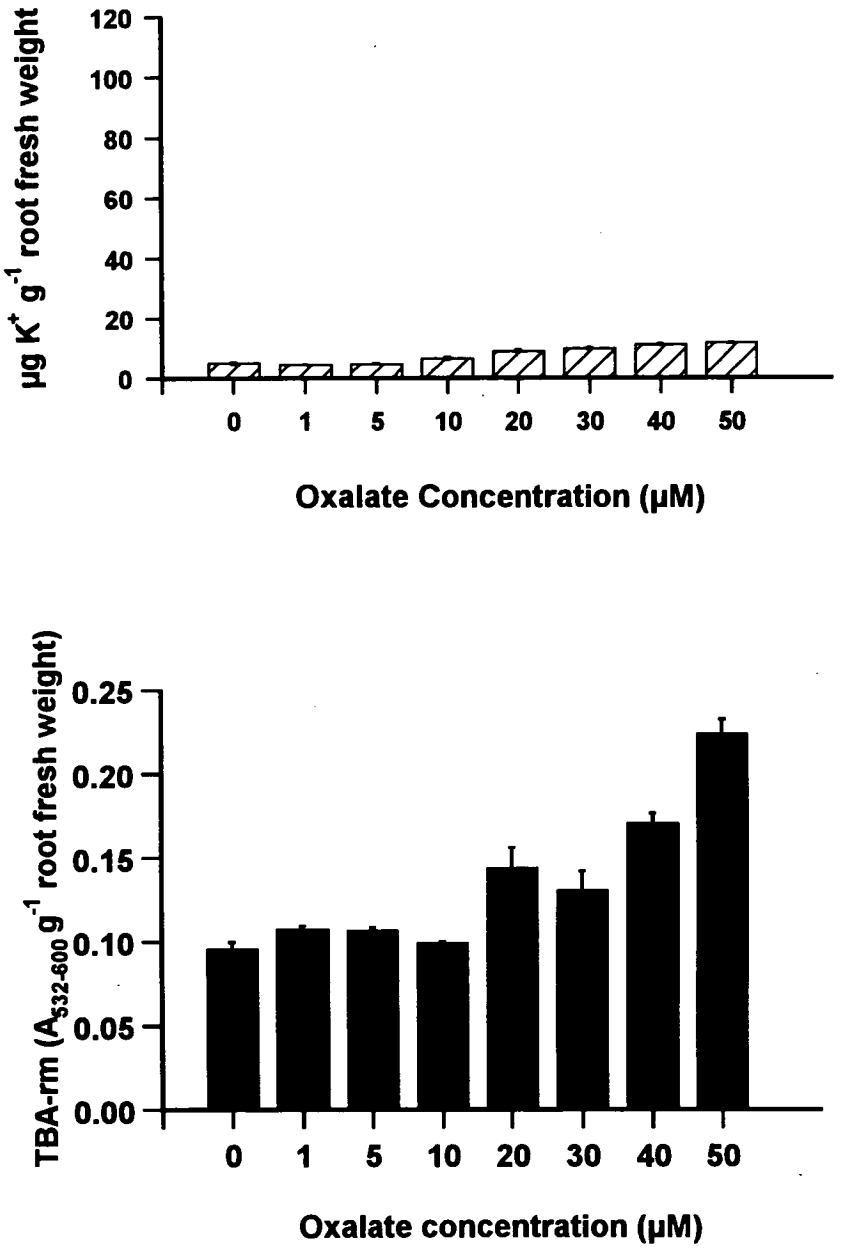


Fig. 6.27. Effect of different concentrations of oxalate on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

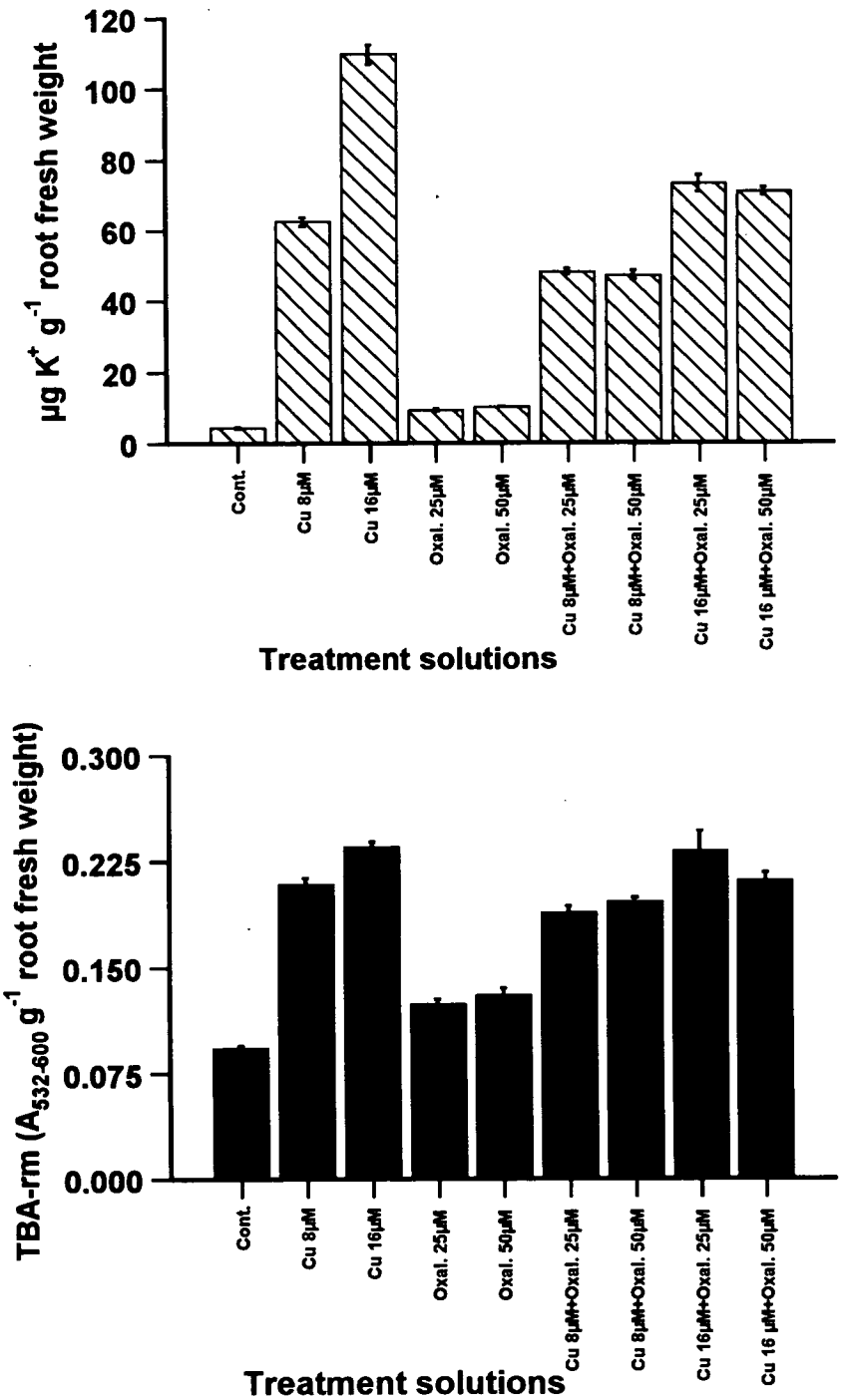


Fig. 6.28. Oxalate-induced modification of the effects of copper ion toxicity on the amount of K^+ leaked from, and on the amount of TBA-rm accumulated in, the roots of 15 d seedlings of rice (*Oryza sativa* L.) cv. NIAB 6. Error bar ± SEM, n=3.

and 16 μM copper + 50 μM oxalate solutions was 33 % and 35 % less ($p<0.05$) than the amount of potassium ions leaked from seedlings incubated in the 16 μM copper ion solution. The amount of potassium leaked into the 8 μM copper + 50 μM oxalate solution was the same as the amount of potassium ions leaked into the 8 μM copper + 25 μM oxalate solution, and the amount of potassium ions leaked into the 16 μM copper + 50 μM oxalate treatment solution was only very slightly less than the amount of potassium ions leaked into the 16 μM copper + 25 μM oxalate solution.

The amount of TBA-rm in the roots after incubation with 8 μM copper + 25 μM oxalate and 8 μM copper + 50 μM oxalate was less than the amount of TBA-rm in the roots after they had been incubated in the 8 μM copper. When the roots were incubated in the 16 μM copper + 25 μM oxalate the amount of TBA-rm was similar to that of the roots after they had been incubated in the 16 μM copper. However, when the roots were incubated in 16 μM copper + 50 μM oxalate solution the amount of TBA-rm was 11 % less ($p < 0.05$) than that of the roots of the seedlings incubated in the 16 μM copper solution.

6.6. Discussion

Calcium and Magnesium

Dose-response curves of the mean length of the longest roots of rice seedlings showed maximum root length when the seedlings were grown in buffered Yoshida solution supplemented with 4 mM calcium (Fig. 6.1) or 2 mM magnesium (Fig. 6.8). In order to investigate the ameliorative role of calcium in relation to the toxic effect of lead ions on *Hordeum vulgare* and *Festuca ovina*, Garland and Wilkins (1981) used 0.42, 1.27, 4.24 and 12.72 mM calcium, whereas, Keltjens and Dijkstra (1991) used 2.5 and 5.0 mM calcium and magnesium to alleviate aluminium ion toxicity in wheat seedlings. Therefore, on the basis of the results of dose-response curve and the findings of other workers, 5 mM and 10 mM calcium, and 4 mM and 8 mM magnesium were used in the present study to investigate the amelioration of copper ion-induced toxicity in rice seedlings.

The addition of copper to the nutrient solution resulted in reduced root and shoot lengths and lower fresh and dry weights of the seedlings at all levels of calcium and magnesium. Seedling growth was stimulated at 5 mM calcium because the basic Yoshida solution was apparently deficient in calcium, but the percentage copper ion-induced inhibition was smaller at 10 mM calcium than at 5 mM calcium i.e.

amelioration was greater at 10 mM calcium. This result is in line with the findings of Garland and Wilkins (1981), who observed an increase in the root elongation of seedlings in lead ion solutions when the concentration of calcium was raised to 12.72 mM. The effect of 8 μM copper on shoot length, and on shoot fresh and dry weight of the seedlings was greater than the effect of the same treatment observed in Section 4.4.2 (Figs. 4.10, 4.11 & 4.12). The reason for this difference is not known. Magnesium at 4 mM or 8 mM concentration showed a slight inhibition of root elongation of the seedlings. When Keltjens and Dijkstra (1991) grew wheat seedlings in 0.25 mM, 2.5 mM and 5.0 mM magnesium they found that the root length of the seedlings was inhibited significantly with increasing external concentration of magnesium. Amelioration of copper ion-induced inhibition in root elongation was greater in the seedlings grown at 8 mM magnesium than in the seedlings grown at 4 mM magnesium, the amelioration being greater at lower concentrations of copper. The ameliorative effect of 8 mM magnesium in relation to copper ion-induced toxicity appears to be greater on shoot length and shoot fresh and dry weight than on root length and root fresh and dry weight of the seedlings.

While some degree of amelioration due to calcium or magnesium was observed however, growth was never completely restored to that of the seedlings which were grown in control solutions (1 μM copper). The ameliorative effects of calcium on copper ion-induced toxicity in relation to root length and root dry weight of rice seedlings were greater than those of magnesium. This result agrees with the findings of Keltjens and Dijkstra (1991) who found that the root growth of aluminium stressed seedlings was ameliorated more by 5 mM calcium in the nutrient solution than by the same concentration of magnesium in the nutrient solution. However, in the present investigation magnesium appeared more ameliorative than calcium to shoot length and shoot fresh and dry weight. Keltjens and Dijkstra (1991) also reported that in relation to shoot dry weight 5 mM magnesium in the nutrient solution gave more protection to wheat seedlings against aluminium toxicity than that which was given by calcium. Robertson (1985) applied different concentrations of calcium and magnesium in order to alleviate nickel toxicity in maize seedlings and found that magnesium at low levels and calcium at rather higher levels can completely protect maize seedlings against nickel toxicity.

At all levels of calcium and magnesium, accumulation of lipid peroxidation products in the roots of the seedlings was observed. The amount accumulated was slightly higher in the roots incubated with different concentrations of magnesium ions

than in the roots which were incubated with calcium ions. Ronald and Söderhäl (1985) and Castillo (1992) reported that peroxidases in cells could be activated by enhanced cytoplasmic concentrations of calcium and magnesium, consequently causing peroxidation of the membrane lipids. As the roots in the present investigation were incubated in a solution of calcium chloride or magnesium chloride alone i.e. not in a balanced nutrient solution, ionic stress caused by these ions might have resulted in increased lipid peroxidation. The lipid peroxidation of the plasmamembrane of the root cells due to calcium or magnesium apparently did not affect the permeability of the plasmamembrane because none of the concentrations of calcium and magnesium investigated caused any higher amount of potassium ion leakage than that observed in the control seedlings. When the concentration of magnesium in the external solution was increased, there was a slight corresponding increase in the amount of potassium ions leaked. However with increasing concentrations of calcium the amount of potassium leaked in the external solution was diminished. In contrast to the present difference in calcium and magnesium in causing leakage of potassium, Grant and Racz (1990), investigating the role of calcium and magnesium on solute leakage from the roots of barley, reported that calcium and magnesium had similar effects on electrolyte leakage. Mengel and Kirkby (1979) discussed the role of calcium in decreasing membrane leakage, and concluded that higher concentrations of calcium in the external solution cause an increase in the uptake and retention of potassium ions by roots. It may therefore be concluded that the increased lipid peroxidation observed when roots of rice seedlings were incubated in a calcium chloride solution or a magnesium chloride solution could be the result of peroxidation of internal membranes caused by enhanced activity of peroxidases and not the result of peroxidation of the plasmamembrane of the root cells.

The ameliorative effect of calcium ions on the amount of potassium ion leakage was slightly greater than that of magnesium ions. At 10 mM calcium, the amount of potassium ions leaked into the external solution was 50 % less than when the external medium contained the same concentration of copper without calcium. Zhao *et al.* (1987) reported that 3.7 mM calcium partly alleviated aluminium effects on the membrane permeability of cells of the root cortex in *Quercus rubra*. Stevenink (1965) and Kawasaki *et al.* (1973) reported that magnesium was much less effective than calcium in reducing leakage of solutes from roots. However, in contrast, Bisson (1984) investigating the role of calcium on electrogenic pump and passive permeability of the plasmamembrane of *Chara corallina*, found that magnesium was

generally as effective as calcium in restoring membrane function. The amount of lipid peroxidation products accumulated in the roots of rice seedlings when they were incubated in a solution having calcium in combination with copper was similar to that accumulated when magnesium was present in combination with copper. No amelioration, by calcium or magnesium, of the effect of copper on the lipid peroxidation was observed. The effects of calcium and magnesium ions on the accumulation of lipid peroxidation products in the roots appeared in fact to be synergistic with those of copper ions.

It is not possible to correlate calcium and magnesium amelioration of the effects of copper ions in relation to plasmamembrane leakage and lipid peroxidation with the amelioration of seedling growth brought about by these divalent cations. However, it appears that calcium and probably magnesium are essential to maintain the structural integrity of the plasmamembrane (Kirkby and Pilbeam 1984). Copper, by reacting with the plasmamembrane, causes lipid peroxidation of the membranes and lesions are formed, thus allowing more copper ions to enter the cells and enabling the efflux of solutes to take place at the same time (De Vos *et al.* 1989). This effect can cause loss in cell turgor and reduced cell elongation and cell wall plasticity. The addition of calcium and magnesium to the solution ameliorated leakage of potassium ions to some extent but did not ameliorate lipid peroxidation. Calcium and magnesium ions at high concentrations may compete with copper for the common binding sites on the plasmamembrane and/or on the cell wall thus rendering the copper ions less damaging. In this way, calcium and magnesium ions may be able to prevent to some extent the damage done by copper to the plasmamembrane but they are apparently unable to protect the membrane systems in the root cells. Keltjens and Dijkstra (1991) concluded that both calcium and magnesium have the ability to exclude aluminium from root adsorption and absorption sites and they found magnesium much more effective than calcium.

Citrate and Oxalate

Dose-response curves of the mean length of the longest roots of rice seedlings showed that root elongation was enhanced when the concentration of citrate (Fig. 6.15) or oxalate (Fig. 6.22) was increased in the nutrient solution from 1 μM to 50 μM. Citric acid and oxalic acid were selected for amelioration of copper ion-toxicity because they exist in acid soils or flooded soils rich in organic matter (Bruckert 1970). Both of these acids are considered to be strong complexers of toxic metal ions. The 25 μM and 50 μM concentration of each acid was chosen within the

mid-range of reported values for acid soil solutions as has been mentioned by Hue *et al.* (1986) and Suthipradit *et al.* (1990).

When citrate or oxalate were incorporated into the nutrient solution the root and shoot lengths and the fresh and dry weights of the seedlings were greater than those of seedlings grown without the addition of these acids. Addition of copper ions to the nutrient solutions at all levels of citrate and oxalate resulted in seedlings which had shorter roots and shoots and lower fresh and dry weights than seedlings grown without the addition of copper. The amelioration of copper ion-induced inhibition of root and shoot elongation and of dry matter accumulation was greater at 50 μM concentration of both acids than at 25 μM. The inhibition in root elongation of the seedlings grown in 8 μM copper relative to those grown in 1 μM copper was ameliorated by more than 90 % by citrate and by oxalate in the nutrient solution. Citrate and oxalate effected amelioration of the inhibition of seedling growth caused by 16 μM copper solution, however, neither of the acids brought about any amelioration when seedlings were grown in 32 μM copper. Hue *et al.* (1986) reported that at 50 μM citric acid and oxalic acid, the acids are most effective in alleviating the toxic effects of 18.5 μM aluminium on root elongation in cotton seedlings. They reported a complete amelioration of the toxic effect of 18.6 μM aluminium, the concentration which killed the roots of cotton seedlings in the absence of either of the acids in the nutrient solution. However, Suthipradit *et al.* (1990) reported that 50 μM oxalic acid failed to show any protective effect against aluminium toxicity in seedlings of soybean, cowpea and green gram.

The ameliorative effect of citrate in relation to copper-induced inhibition of root elongation in rice seedlings was similar to that of oxalate. However, the ameliorative effects of oxalate on root and shoot fresh weights were slightly greater than those of citrate. It is assumed that in seedlings grown in the presence of citrate and oxalate, high concentrations of these ions were present inside the cells, thus enabling internal sequestration of copper ions to take place. The computer simulation analysis showed that about 80 % of the added citrate or oxalate was complexed with both calcium and magnesium and that there was no effect of either of the acids on the speciation of copper ions in the nutrient solution, more than 95 % of added copper being complexed with EDTA. This suggests that it was some sort of internal detoxification which was carried out by citrate or oxalate rather than some sort of ionic competitive effect for absorption sites.

Lipid peroxidation products were accumulated in the roots when they were incubated with a range of concentrations of citrate or oxalate. Lipid peroxidation of the membranes of root cells was slightly higher when roots were incubated with the higher concentrations of oxalate than with the higher concentrations of citrate. Ionic stress caused by these ions might have resulted in an increased lipid peroxidation. However, lipid peroxidation of the plasmamembrane of the root cells due to citrate or oxalate, apparently did not affect the permeability of the plasmamembrane because the amount of potassium ions leaked in the presence of citrate and oxalate was very small. There is no published information available about the effect of citrate or oxalate on the process of lipid peroxidation in the plasmamembrane of the root cells. In the present investigation, roots of the seedlings were incubated in solution containing only citrate or oxalate i.e. not Yoshida solution supplemented with citrate or oxalate, therefore it is possible that large amounts of these ions were taken up by the roots, resulting in some sort of internal ionic imbalance, and that this ionic stress led to increased lipid peroxidation.

For all levels of copper, the presence of citrate and oxalate reduced the amount of potassium ions leaked from the roots of the seedlings into the external solution by at least 50 %. The ameliorative effect of citrate on the copper ion-induced potassium ion leakage from the roots of the seedlings was greater when the roots were incubated in different combinations of copper with citrate than when the roots were incubated in different combinations with oxalate. Harmens *et al.* (1994) studied the role of low molecular weight organic acids in the mechanism of increased zinc tolerance in *Silene vulgaris* and concluded that citrate or oxalate, might play an important role in the sequestration of toxic levels of zinc, because of their higher concentrations inside the plant. It is likely that in the present set of experiments, where there was no EDTA in the solution, copper was chelated with citrate or oxalate. Therefore this chelated form of copper, when taken up, was sequestered inside the root cells, rendering it less toxic. However, the actual mechanism of this ameliorative role is yet to be elucidated. Suthipradit *et al.* (1990) measured the amount of monomeric aluminium i.e. the toxic form in solutions with oxalic acid and reported that 50 μM oxalic acid was able to complex 30 % of the 50 μM aluminium, and that the remaining 70 % of aluminium ions was toxic enough to affect the root growth of soybean, cowpea and green gram seedlings. The measurement of lipid peroxidation products accumulated in the roots of rice seedlings during incubation in a solution having citrate in combination with copper was similar to that when oxalate was present in combination with copper. The amount of lipid peroxidation products

accumulated in the roots when citrate and oxalate were present in combination with copper was almost equal to or only slightly less than that which was observed when only copper was present in the incubation medium. The citrate and oxalate together did reduce potassium leakage but not lipid peroxidation, therefore, it might be concluded that citrate and oxalate can protect plasmamembrane but not internal membranes. Neverthless, the effect of citrate and oxalate in ameliorating copper ion-induced leakage and lipid peroxidation of the plasmamembrane of the root cells cannot be fully explained.

The protection provided by citrate and oxalate was greater than that provided by calcium and magnesium. Root elongation of the seedlings grown in Yoshida nutrient solutions supplemented with citrate or oxalate was less inhibited by copper ion toxicity than seedlings grown in the Yoshida nutrient solution supplemented with calcium or magnesium ions. The computer simulation predicted that at each concentration of copper, more than 95 % of the copper in the supplemented nutrient solution was present in the form of a complex with EDTA. The very small fraction of copper present in ionic form might not be toxic enough to have a deleterious effect on root growth. It appears therefore that agents which apparently bring about the internal detoxification of copper ions are more effective than those which act by excluding copper ions from the binding sites on the plasmamembrane.

7. GENERAL DISCUSSION AND SUGGESTIONS FOR FUTURE WORK

Pollution of agricultural lands by metal ions is becoming a global problem. Cereals are the dietary mainstay for the majority of the world's people. Due to many natural disasters around the world and to very rapidly increasing populations, the demand for cereals is increasing enormously. Therefore it is important to investigate the response of cereals to toxic metal ions and to understand the mechanisms of metal ions toxicity. In order to get more precise information about the effects of metal ions on plant growth, especially when the metal ions are toxic in micro molar concentrations, studies are mostly carried out under controlled environmental conditions using solution culture techniques. This technique allows the maintenance of very small concentrations of metal ions in the root medium, and exposes seedlings to a uniform concentration of the metal ions applied.

Germination and seedling growth are critical stages in the life cycle of a crop plant as they determine the density of the final crop stand. The effect of copper, lead and zinc on germination and seedling growth of wheat, barley and rice was tested under controlled environment conditions. Generally the process of germination appeared insensitive to the effect of toxic metal ions. At low concentrations of copper, lead and zinc, the percentage germination of wheat, barley and rice was merely retarded, however $CuSO_4$ at 10 mM concentration was toxic enough to inhibit the process of germination. Of the three species, rice appeared to be the most sensitive to the copper ions. The main difficulty in comparing the results of seed germination with results of other workers is the lack of uniformity in the criteria used to classify seeds as germinated. The physiological definition of germination as given by Bewley and Black (1994) states that germination begins with water uptake by the seed and ends with the start of elongation by the embryonic axis, usually the radicle and, on the emergence of the radicle, seed is considered to be germinated. According to this definition one must be very precise in identifying the seeds in which emergence has taken place. However, in order to have a more practical and easily recognisable criterion, a certain minimum radicle length is often used (Kaur and Duffus 1989).

It is the growth of the seedling and not the process of germination which is the most sensitive stage in the plant's life. Growing roots and shoots may be susceptible to metal ion concentrations which do not have any effect on seed germination. Seedling growth tests were performed using a modified form of the

rolled paper towel test (Perry 1977). The seedlings were grown for 7 d in different concentrations of metal ions only. Copper ions produced a stronger effect on seedling growth than lead and zinc. The root elongation of the seedlings appeared more sensitive than shoot elongation to metal ion toxicity. There was no relationship between the concentrations of metal ions which inhibited the process of seed germination and the concentrations which were toxic to seedling growth. The concentrations of metal ions which caused toxic effects on seedling growth in the rolled paper towel test were quite high compared to the concentrations which have been used in nutrient culture experiments.

Further work was carried out by using a nutrient culture technique in order to determine the minimum concentration of copper ions toxic to the growth of the rice seedlings, and the physiological effects of that concentration on roots. Rice was selected because it is one of the major cereals and it does not need aerated nutrient solution for the growth of the seedlings. Two cultivars of rice, Basmati 37-a salinity-sensitive cultivar and NIAB 6-a salinity tolerant cultivar, were used in the experiment to investigate the basis of metal ion tolerance. A copper ion concentration of 8 μM in the nutrient solution was found to be toxic enough to inhibit the root elongation of the seedlings, however, significant inhibition in root elongation was observed at 16 μM copper in both the cultivars. Roots of the seedlings appeared as the major organs for the accumulation of high concentrations of copper ions. Both the cultivars differed in their response to copper ions. Though the differences were small they were repeatable. The results showed that there is a possibility of identifying tolerant and sensitive cultivars of rice. In future, a thorough screening should be done in order to find out metal-tolerant genotypes in crop plants. Those genotypes could then be used to understand the physiological and genetic basis of metal ion tolerance . Once this is known the appropriate genes could be incorporated into sensitive genotypes.

The form in which applied copper ions were present in the nutrient solution was determined by the Computer simulation programme GEOCHEM-PC. The simulation results predicted that about 99 % of the applied copper was present in the solution as EDTA complex and no precipitation was predicted. This copper EDTA complex is a readily available form of copper. It appears therefore, that with increased concentration of copper more of the EDTA copper complex was present around the roots and perhaps more of it was taken up by the roots. Once inside the

root some configurational change apparently takes place which causes the toxic effects of copper.

There is a strong interaction between the pH of the medium and metal ion toxicity. This fact is well established for aluminium and cadmium ions, which become more toxic at low pH. Copper ion-induced toxicity to the growth of rice seedlings was investigated at pH 5.5, 5.0 and 4.5. To generate greater differences between the treatment means, the concentration of copper in the treatments was doubled. Root length was least at pH 4.5 however, the percentage inhibition in root elongation of the seedlings due to copper ion-toxicity relative to the respective control was greater at pH 5.5 than at pH 5.0 or 4.5. The amount of chlorophyll in the uppermost fully expanded leaves was also measured. The results showed that the amount of total chlorophyll in the leaves was not affected by pH of the solution, however 8 μM copper caused more than 50 % inhibition in the amount of chlorophyll. Further work in this line must be carried out to investigate the effects of high pH on copper ion-induced toxicity in seedlings. It would be of much importance especially for the soils which have near neutral pH.

The effect of copper ions on the root physiology of both the cultivars was investigated in relation to potassium ion leakage from and lipid peroxidation in the root cells. This was a short term treatment in which the whole intact root systems of the seedlings were incubated with solutions of $CuSO_4$ only. A copper ion concentration of 4 μM, which was non toxic to seedling growth when present in nutrient solution, within a short period of time, caused a significant amount of potassium leakage from the roots and the accumulation of lipid peroxidation products inside the roots was also increased. NIAB 6, a cultivar which in regard to root growth was slightly more tolerant of copper than Basmati 370, showed slightly less potassium leakage than Basmati 370. The incubation solution consisted of only $CuSO_4$, therefore, the toxic effect of copper ions to potassium leakage and lipid peroxidation could be attributed to high adsorption of copper ions on the plasmamembrane of the root cells, causing peroxidation of membrane lipids. The plasmamembrane integrity was damaged in this way and this resulted in leakage of potassium ions and other solutes from the root cells. These results cannot be used to explain the effects of copper ions on seedling growth because in the seedling growth experiments, the roots were in a complete nutrient solution containing EDTA in addition to the different concentrations of copper ions. Even at the highest concentration of copper ions a very small fraction of that copper was present in ionic

form. The roots were however, exposed to the copper containing nutrient solutions for a long time, whereas in the physiological study, where all the copper was present in ionic form, the roots were exposed only for a short period of time. Thus the findings of this experiment revealed how copper ion toxicity is implicated in the inhibition of root growth in seedlings. More work should be carried out to investigate the effect of copper ions on potassium leakage and lipid peroxidation, when EDTA is present in the incubation medium. A time course study of potassium ion leakage should be carried out with shorter time intervals to investigate whether the effect of copper ions is time-dependent or dose-dependent and an estimation of the amount of different other solutes leaked along with potassium should be carried out. Studies on copper uptake kinetics by the roots with and without EDTA in the solution are required.

Further observations were made in order to investigate how the physiological effects of copper ion toxicity in roots are translated into morphological effects. It was observed that inhibition of root elongation was accompanied not only by inhibition in the elongation of root hairs but also by failure of root hair development. The effect became more pronounced with the prolongation of the copper ion treatment. Moreover, the formation of root hairs took place at a distance further away from the root-apex. After 12 d of treatment the whole root was found to be hairless. Root hair length is a prime factor in increasing nutrient uptake and it is generally accepted that root hair development and turnover is a major means for establishing a well-developed and efficient nutrient and water absorbing root system. Thus, the copper ion induced inhibition in the elongation of the root hair and reduction in root hair intensity is likely to have a negative effect on the capacity of the roots to absorb water and nutrients. Further work must be carried out to understand the mechanisms of copper ion-induced inhibition in development and growth of root hairs.

When roots were grown in toxic concentrations of copper ions, lateral branches were close to each other and nearer to the root-apex. This could be due to change in hormonal action inside the root, leading to smaller and or fewer root cells in between the adjacent root primordia. However, the emergence of lateral branches showed that some level of cell division continues even though cell elongation was severely inhibited by copper ions. More experimental work is needed to find out the effect of copper ions on the mitotic index of the root-apex.

It was difficult to see any change on the surface of the root using fresh specimens, as the root surface was either completely covered by a thick blanket of root hairs or by a thick layer of mucilage. Dehydration and fixation is a long and difficult process, and during the course of specimen preparation, the epicuticular waxes of the root surface are dissolved. The most convenient approach, therefore, was to make a replica of the fresh roots. A replica technique was developed and applied successfully to investigate the effect of copper ions on the root epidermis. Scanning electron micrographs of the replicas enabled the effects of copper ions on the cells of the root epidermis to be quantified. The length of the epidermal cells of the roots which were grown in the copper ion solution was significantly smaller than the length of the epidermal cells of the control roots. This difference was more when the seedlings were grown in the copper solution for a longer period. It was not only the elongation of the cells which was inhibited but the width of the cells was also significantly less in the roots of the seedlings grown in the copper ion solution than in those which were grown in the control solution. The process of cell elongation is a very complex process and it depends upon turgor maintenance, and on the loosening and synthesis of cell wall materials possibly under the control of growth regulators. Copper inhibits one or all of these processes and thus ultimately inhibits root growth. Further work in this line should be focused on cytochemical aspects to see where in roots excess copper ions are accumulated, and what sort of ultrastructural changes in intracellular organelles are brought about by the accumulation of copper ions.

After determining the concentrations of copper ions toxic to seedling growth, and investigating some of the physiological and morphological changes in roots brought about by copper ions, an attempt was made to use different ameliorants to protect the seedlings from th toxic effects of copper. The inorganic ameliorants calcium and magnesium, and the organic ameliorants, citrate and oxalate were included with copper in the nutrient solution. The inclusion of 10 mM calcium or 8 mM magnesium in the nutrient solution decreased the copper ion-induced percentage inhibition relative to the respective controls. However in root growth, ameliorative effect of calcium was greater than that of magnesium, whereas, in shoot growth magnesium appeared more ameliorative than calcium. The reason could be that calcium is more involved in root elongation and growth, whereas magnesium has a role in chlorophyll synthesis (Marschner 1986). If calcium and magnesium were applied in different combinations it might be possible to achieve further amelioration of copper toxicity. The effect of calcium and magnesium on copper ion-induced changes in root morphology also needs to be investigated.

The ameliorative effect of calcium on potassium ion leakage was greater than that of magnesium, and at 10 m M calcium in the external solution, the amount of potassium leaked was 50 % less than when the external solution contained the same amount of copper without calcium. However, the accumulation of lipid peroxidation products in the roots did not show any ameliorative effect of either calcium or magnesium, rather, when calcium or magnesium was present in addition to copper the effect on lipid peroxidation was synergistic. From these results it appears that calcium or magnesium provided a protective role to the plasmamembrane integrity and reduced the amount of potassium leakage, however the lipid peroxidation of the internal membranes remained unchecked. The protective role of these ions is either through a competitive effect with copper ions for binding sites on the plasmamembrane, or through some physiological change in membrane structure which blocked the channels through which efflux of potassium was taking place. The effect of different combinations of calcium and magnesium on potassium leakage and lipid peroxidation of the membranes should be investigated. These investigations should be carried out with EDTA also present in the incubation medium so that the results can be correlated with effects on seedling growth.

When citrate and oxalate were incorporated into the nutrient solution at a concentration of 50 μM, both ameliorated the toxic effects of 8 μM copper ions, though seedling growth was not completely restored. Both citrate and oxalate showed a similar amelioration of the effects of copper on seedling growth. The results of GEOCHEM-PC simulation predicted that more than 80 % of applied citrate or oxalate in the nutrient solution was present complexed with calcium and magnesium. At the same level more than 95 % of the applied copper is complexed with EDTA. Therefore, the application of citrate or oxalate does not show chelation of copper ions in the external solution. There is no information in the literature about the mechanisms influencing the uptake of citrate or oxalate by the roots and their fate within the plant. However, the assumption is that when these ions are present in high concentration they are taken up by plant roots. Once they are present in high concentrations inside the root they may cause sequestration of copper ions. Internal sequestration of metal ions by high concentrations of citrate or oxalate is known (Harmens *et al.* 1994). However, further work needs to be carried out to investigate whether citrate or oxalate is taken up by the roots, and if so, how high concentrations of these ions can sequester copper ions inside the root but not in the nutrient solution.

The investigations in relation to potassium ion leakage and lipid peroxidation of the plasmamembrane revealed that the amount of potassium ions leaked from the roots into the external solution in the presence of citrate or oxalate was less than 50 % of that leaked when the external solution contained the same amount of copper in the absence of citrate or oxalate. Citrate showed greater amelioration of potassium leakage than oxalate. Citrate and oxalate caused an increase in lipid peroxidation. However, the presence of either citrate or oxalate showed only a very small ameliorative effect on the copper ion induced accumulation of lipid peroxidation products in the roots. The way in which increase in lipid peroxidation is brought about by citrate or oxalate has yet to be investigated. The effects of citrate and oxalate on copper ion-induced changes in root morphology also needs to be determined.

In summary, copper at concentrations as low as 8 μM, inhibited seedling growth. The effect of copper ions on root physiology and morphology was evident at even lower concentrations and after shorter times of exposure. The application of calcium, magnesium, citrate or oxalate could mitigate these toxic effects of copper to some extent, but seedling growth was never completely restored to that of the seedlings which were grown in control solutions. As metal ions in the soil are highly immobile and they stay mostly in the plough layer, application of these ameliorants to allow the roots of seedlings to grow through that toxic zone in soil would help seedlings to become established. This would ultimately be helpful in putting marginally polluted soils back to production.

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Appendix I

Detection Limits of Metals by ICAP 61E, Plasma Emission Spectrometer

Elements	Concentration Expressed As	Detection Limit
S	$mg\ L^{-1}$	0.20
Mg	$mg\ L^{-1}$	0.10
Na	$mg\ L^{-1}$	0.35
K	$mg\ L^{-1}$	2.20
Ca	$mg\ L^{-1}$	0.05
Al	$\mu g\ L^{-1}$	80.00
Fe	$\mu g\ L^{-1}$	230.00
Mn	$\mu g\ L^{-1}$	4.00
Cu	$\mu g\ L^{-1}$	11.00
Zn	$\mu g\ L^{-1}$	9.00
P	$\mu g\ L^{-1}$	220.00
As	$\mu g\ L^{-1}$	200.00
Cd	$\mu g\ L^{-1}$	14.00
Cr	$\mu g\ L^{-1}$	13.00
Ni	$\mu g\ L^{-1}$	30.00
Pb	$\mu g\ L^{-1}$	110.00
Se	$\mu g\ L^{-1}$	350.00
B	$\mu g\ L^{-1}$	45.00
Ba	$\mu g\ L^{-1}$	4.00

Appendix II

Composition of Yoshida Nutrient Solution

	Chemicals	Weight in g L-1
	Macronutrients	
1	NH_4NO_3	91.400
2	K_2SO_4	71.400
3	KH_2PO_4	92.400
4	K_2HPO_4	17.100
5	$CaCl_2.6H_2O$	175.000
6	$MgSO_4.7H_2O$	324.000
	Micronutrients	
7	$MnCl_2.4H_2O$	1.500
8	$NH_4MoO_4.4H_2O$	0.074
9	H_3BO_3	0.930
10	$ZnSO_4.7H_2O$	0.035
11	$CuSO_4.5H_2O$	0.030
12	FeNa EDTA	10.500

Dissolve number 3 & 4 together to prepare one stock solution.
Dissolve number 7 to 11 together to prepare one stock solution.
Keep number 12 stock solution in dark and cool place.

Take 1.25 mL from each stock solution and make up the volume to one litre that will be full strength Yoshida solution (Yoshida *et al.* 1976). Adjust pH of the solution to 5.5.

Appendix III

Acetate Buffer

Solution A.

100 mM Acetic acid was prepared by taking 6.05 ml of glacial acetic acid in 1 L flask and making up the volume by adding distilled water.

Solution B.

100 mM Sodium acetate was made by dissolving 8.2 g of sodium acetate in 1 L disstiled water.

Both solutions were mixed in the following different proportion and diluted to 100ml by adding distilled water. The pH of the buffer (pH b) thus measured is given in the following table.

A (ml)	B (ml)	pH b
45	5	3.28
40	10	3.60
37	13	3.70
35	15	3.95
33	17	4.02
30	20	4.11
25	25	4.32
20	30	4.53
15	35	4.70
10	40	4.97
4	46	5.47
00	50	6.50

Appendix IV

Calculations don by GEOCHEM-PC for 5 mM Ca and 16 µM copper in Complete Yoshida Nutrient solution.

Metals	Distribution	Ligands	Distribution
Ca	90.10 % as free metal ion	SO_4	71.37 as a free ligand ion
	8.28 % complexed with SO_4		22.81 % complexed with Ca
	0.06 % complexed with Cl		5.10 % complexed with Mg
	1.55 % complexed with PO_4		0.66 % complexed with K
Mg	92.07 % as a free metal ion		0.04 % complexed with Mn^{2+}
	6.72 % complexed with SO_4		0.01 % complexed with H^+
	0.57 % complexed with Cl	Cl	99.85 as a free ligand ion
	0.64 % complexed with PO_4		0.04 % complexed with Ca
K	99.15 % as a free metal ion		0.08 % complexed with Mg
	0.68 % complexed with SO_4		0.03 % complexed with K
	0.18 % complexed with Cl	PO_4	9.58 % complexed with Ca
Na	98.68 % as afree metal ion		1.09 % complexed with Mg
	0.42 % complexed with SO_4		1.67 % in solid form with Fe^{3+}
	0.90 % complexed with Cl		87.65 % complexed with H^+
Fe^{3+}	46.12 % in solid form with PO_4	EDTA	0.07 % complexed with Ca
	53.87 % complexed with EDTA		53.87 % complexed with Fe^{3+}
Mn^{2+}	88.89 % as a free metal ion		0.16 % complexed with Mn^{2+}
	8.17 % complexed with SO_4		45.61 % complexed with Cu^{2+}
	2.34 % complexed with Cl		0.30 % complexed with Zn
	0.60 % complexed with EDTA	$B(OH)_4$	0.02 as a free ligand
Cu^{2+}	0.19 % as a free metal ion		99.98 % complexed with H^+
	0.02 % complexed with SO_4	MoO_4	96.12 as a free ligand
	99.78 % complexed with EDTA		3.88 % complexed with H^+
Zn	26.10 % as a free metal		
	2.40 % complexed with SO_4		
	0.43 % complexed with Cl		
	0.99 % complexed with PO_4		
	69.97 % complexed with EDTA		
	0.10 % complexed with OH^-		

Appendix V

Calculations don by GEOCHEM-PC for 4 mM Mg and 16 µM copper in Complete Yoshida Nutrient solution.

Metals	Distribution	Ligands	Distribution
Ca	99.01 % as free metal ion	SO_4	79.52 as a free ligand ion
	9.89 % complexed with SO_4		4.62 % complexed with Ca
	0.05 % complexed with Cl		15.00 % complexed with Mg
	1.72 % complexed with PO_4		0.77 % complexed with K
	0.33 % complexed with OAc		0.02 % complexed with Na
Mg	90.31 % as a free metal ion		0.04 % complexed with Mn^{2+}
	8.06 % complexed with SO_4		0.02 % complexed with H^+
	0.48 % complexed with Cl	Cl	99.76 as a free ligand ion
	0.72 % complexed with PO_4		0.19 % complexed with Mg
	0.42 % complexed with OAc		0.03 % complexed with K
K	99.08 % as a free metal ion	PO_4	1.76 % complexed with Ca
	0.77 % complexed with SO_4		2.94 % complexed with Mg
	0.15 % complexed with Cl		0.83 % in solid form with Fe^{3+}
Na	98.73 % as afree metal ion		94.46 % complexed with H^+
	0.48 % complexed with SO_4	OAc	81.77 as a free ligand ion
	0.76 % complexed with Cl		0.66 % complexed with Ca
	0.02 % complexed with OAc		3.38 % complexed with Mg
Fe^{3+}	22.93 % in solid form with PO_4		14.19 % complexed with H^+
	77.06 % complexed with EDTA	EDTA	0.02 % complexed with Ca
Mn^{2+}	86.85 % as a free metal ion		77.06 % complexed with Fe^{3+}
	9.76 % complexed with SO_4		0.24 % complexed with Mn^{2+}
	1.99 % complexed with Cl		22.35 % complexed with Cu^{2+}
	0.52 % complexed with OAc		0.33 % complexed with Zn
	0.89 % complexed with EDTA	$B(OH)_4$	0.02 as a free ligand
Cu^{2+}	0.12 % as a free metal ion		99.98 % complexed with H^+
	0.02 % complexed with SO_4	MoO_4	96.00 as a free ligand
	99.85 % complexed with EDTA		4.00 % complexed with H^+
Zn	18.99 % as a free metal		
	2.13 % complexed with SO_4		
	0.27 % complexed with Cl		
	0.82 % complexed with PO_4		
	0.28 % complexed with OAc		
	77.41 % complexed with EDTA		
	0.08 % complexed with OH^-		

Appendix VI

Calculations don by GEOCHEM-PC for 25 mM oxalate and 16 μM copper in Complete Yoshida Nutrient solution.

Metals	Distribution	Ligands	Distribution
Ca	83.91 % as free metal ion	SO_4	84.90 as a free ligand ion
	12.96 % complexed with SO_4		5.93 % complexed with Ca
	0.01 % complexed with Cl		8.17 % complexed with Mg
	1.87 % complexed with PO_4		0.92 % complexed with K
	1.24 % complexed with Oxal		0.06 % complexed with Mn^{2+}
Mg	87.79 % as a free metal ion		0.02 % complexed with H^+
	10.77 % complexed with SO_4	Cl	99.87 as a free ligand ion
	0.11 % complexed with Cl		0.09 % complexed with Mg
	0.82 % complexed with PO_4		0.03 % complexed with K
	0.52 % complexed with Oxal	PO_4	1.92 % complexed with Ca
K	99.02 % as a free metal ion		1.39 % complexed with Mg
	0.94 % complexed with SO_4		1.66 % in solid form with Fe^{3+}
	0.03 % complexed with Cl		95.03 % complexed with H^+
Na	99.24 % as afree metal ion	Oxal	14.50 as a free ligand ion
	0.60 % complexed with SO_4		49.56 % complexed with Ca
	0.16 % complexed with Cl		34.26 % complexed with Mg
Fe^{3+}	45.82 % in solid form with PO_4		0.60 % complexed with Mn^{2+}
	54.17 % complexed with EDTA		0.40 % complexed with Cu^{2+}
Mn^{2+}	84.31 % as a free metal ion		0.02 % complexed with Zn
	13.02 % complexed with SO_4		0.66 % complexed with H^+
	0.43 % complexed with Cl	EDTA	0.01 % complexed with Ca
	1.57 % complexed with Oxal		54.17 % complexed with Fe^{3+}
	0.66 % complexed with EDTA		0.18 % complexed with Mn^{2+}
Cu^{2+}	0.16 % as a free metal ion		45.34 % complexed with Cu^{2+}
	0.03 % complexed with SO_4		0.30 % complexed with Zn
	0.60 % complexed with Oxal	$B(OH)_4$	0.02 as a free ligand
	99.20 % complexed with EDTA		99.98 % complexed with H^+
Zn	22.25 % as a free metal	MoO_4	95.66 as a free ligand
	3.44 % complexed with SO_4		4.34 % complexed with H^+
	0.07 % complexed with Cl		
	1.10 % complexed with PO_4		
	3.31 % complexed with Oxal		
	69.72 % complexed with EDTA		
	0.10 % complexed with OH^-		

Appendix VII

Calculations don by GEOCHEM-PC for 25 mM citrate and 16 µM copper in Complete Yoshida Nutrient solution.

Metals	Distribution	Ligands	Distribution
Ca	84.25 % as free metal ion	SO_4	84.89 as a free ligand ion
	13.01 % complexed with SO_4		5.95 % complexed with Ca
	0.01 % complexed with Cl		8.16 % complexed with Mg
	1.88 % complexed with PO_4		0.92 % complexed with K
	0.84 % complexed with Citr		0.06 % complexed with Mn^{2+}
Mg	87.64 % as a free metal ion		0.02 % complexed with H^+
	10.75 % complexed with SO_4	Cl	99.87 as a free ligand ion
	0.11 % complexed with Cl		0.09 % complexed with Mg
	0.82 % complexed with PO_4		0.03 % complexed with K
	0.68 % complexed with Citr	PO_4	1.93 % complexed with Ca
K	99.02 % as a free metal ion		1.39 % complexed with Mg
	0.94 % complexed with SO_4		1.44 % in solid form with Fe^{3+}
	0.03 % complexed with Cl		95.24 % complexed with H^+
Na	99.24 % as afree metal ion	Citr	2.10 as a free ligand ion
	0.60 % complexed with SO_4		33.45 % complexed with Ca
	0.16 % complexed with Cl		45.10 % complexed with Mg
Fe^{3+}	39.71 % in solid form with PO_4		0.04 % complexed with K
	4.91 % complexed with Citr		0.49 % complexed with Mn^{2+}
	55.36 % complexed with EDTA		2.10 % complexed with Cu^{2+}
Mn^{2+}	84.53 % as a free metal ion		0.03 % complexed with Zn
	13.06 % complexed with SO_4		7.04 % complexed with Fe^{3+}
	0.43 % complexed with Cl		9.65 % complexed with H^+
	1.28 % complexed with Citr	EDTA	0.01 % complexed with Ca
	0.70 % complexed with EDTA		55.36 % complexed with Fe^{3+}
Cu^{2+}	0.15 % as a free metal ion		0.19 % complexed with Mn^{2+}
	0.03 % complexed with SO_4		44.14 % complexed with Cu^{2+}
	3.25 % complexed with Citr		0.30 % complexed with Zn
	96.57 % complexed with EDTA	$B(OH)_4$	0.02 as a free ligand
Zn	21.19 % as a free metal		99.98 % complexed with H^+
	3.27 % complexed with SO_4	MoO_4	95.66 as a free ligand
	0.07 % complexed with Cl		4.34 % complexed with H^+
	1.05 % complexed with PO_4		
	4.73 % complexed with Citr		
	69.58 % complexed with EDTA		
	0.10 % complexed with OH^-		